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# REACTIONS OF RABBITS TO NON-HEMOLYTIC STREPTOCOCCI

## III. A Study of Modes of Sensitization

By C. L. DERICK, M.D., C. H. HITCHCOCK, M.D., and HOMER F. SWIFT, M.D.

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(Received for publication, April 3, 1930)

In previous papers (1, 2) we have described the various types of tissue response of rabbits to inoculation with non-hemolytic streptococci. It was shown that reactions following reinfection were conditioned by the route of the original inoculation: if it had been into the tissues—especially into the skin—the subsequent reaction was that of hypersensitiveness, or hyperergy; if into the blood stream, it was that of immunity. For the sake of completeness two other types of reaction were recorded: that of normal animals, which in the majority of instances was followed by a secondary reaction, and that of cachectic rabbits, in which inoculation resulted in a soft, pale lesion smaller than that of normal animals, and not followed by a secondary reaction.

Variability in the response of apparently normal rabbits to inoculation with streptococci is well recognized; and because of this some investigators feel that this species is unsuitable for the study of experimental streptococcus infections. Under ordinary experimental conditions this factor necessitates the introduction of a sufficient number of animals into each experiment to permit of conclusions on a statistical basis. While such a basis is always requisite in biological research the advantages of having to depend upon it as little as possible are obvious.

During our investigations the desirability became more and more evident of ascertaining the best method of rendering a large majority of rabbits satisfactorily hypersensitive to non-hemolytic streptococci in the shortest possible time. This communication gives the details of some of the experiments planned with such an end in view.

## EXPERIMENTAL

Both green-producing and indifferent strains of non-hemolytic streptococci of previously demonstrated sensitizing capacity have been used. From an original stock culture, preserved in the frozen and dried state, sub-stocks in rabbits' blood broth were prepared every 6 to 10 weeks. As the work progressed it seemed that old stock cultures in blood broth gradually lost their sensitizing capacity; more recently, therefore, the blood broth stocks have been renewed from the dried culture every 4 to 6 weeks. The inoculum for the animals was a culture grown for from 18 to 24 hours in 2 per cent rabbits' blood broth to which 0.05 per cent dextrose was added. Both Douglas tryptic broth and beef infusion broth have been used. If the sediment of more than 0.1 cc. of whole culture was to be injected in one place the culture was centrifuged and sufficient of the supernatant fluid was discarded so that 0.1 cc. of the residue represented the amount of growth selected for injection; 0.1 cc. of whole culture was injected if that was the quantity desired: and in the case of the smaller inocula the culture was diluted with broth or Ringer's solution so that 0.05 cc. represented the amount injected. It was found that the intracutaneous injection of 0.05 cc. of some samples of broth gave early evanescent, edematous lesions in some highly sensitized rabbits and occasionally in normal controls; but these lesions never became indurated as did those following injection of culture. Dilution of the culture with Ringer's solution did not give rise to these false reactions; hence only this diluent has been employed recently. All skin lesions were measured daily until marked diminution in size was evident, and the volumes were estimated as previously described (1). The ophthalmic and lethal tests were carried out as previously noted (1); the dose of culture employed for the lethal test was the centrifugate of 4 cc. of blood broth culture per kilogram of rabbit.

To obtain as nearly uniform conditions as possible animals of corresponding weight and color were distributed among the different groups. During the past year we have found that Chinchillas and New Zealand Reds have more consistently become hypersensitive after intradermal inoculation and, on the whole, have had more suitable skins; hence these varieties have been used when obtainable.

Attempts were first made to determine which was the most efficacious in sensitizing animals: one, two or four intracutaneous injections, each with the sediment of 5 cc. of culture. Lesions produced with such large doses frequently broke down and discharged their contents within a few days. Another disadvantage in injecting such large doses was the failure, at times, of the resulting lesions to show clear-cut secondary reactions, whereas those produced by $10^{-1}$ cc. or $10^{-2}$ cc. in the same animals gave distinct evidence of such reactions.

With equivocal secondary reactions in animals treated with large inocula alone it was necessary to resort to ophthalmic and lethal tests to ascertain the state of reactivity. In two or three different experiments the results were so variable that no satisfactory conclusions could be drawn from the exclusive use of these large inocula. It was then observed that rabbits became increasingly sensitive following initial intracutaneous inoculation with the sediment of 5 cc., 1 cc., $10^{-1}$ cc., and $10^{-2}$ cc. of blood broth culture if they subsequently received repeated injections of $10^{-1}$ cc. and $10^{-2}$ cc. at 10-day intervals. In carrying out this procedure so many areas of skin were inoculated that little remained available for subsequent observations; hence it became desirable to devise other modes of sensitization in which less cutaneous damage was inflicted.

*Experiment 1.*—Eighteen rabbits were each inoculated intracutaneously in five areas with the sediment of Culture V110A/0/13, as follows: 5 cc., 5 cc., $10^{-1}$ cc., $10^{-2}$ cc., $10^{-4}$ cc. They were then divided into three equal groups, A, B and C. On the 8th day each animal of Group A received a subcutaneous focus of 5 cc. of agar infected with the sediment of 5 cc. of culture. The animals of Group B were inoculated intramuscularly with $10^{-1}$ cc. and $10^{-2}$ cc. from a similar culture every 5 days from the 5th to the 40th day, and those of Group C with the same doses intracutaneously. The degree of skin reactivity of Group A animals was tested with $10^{-4}$ cc. of culture on the 1st, 15th, 25th, 35th and 40th days, and that of the animals in the other two groups on the 1st, 10th, 20th, 30th and 40th days. The ophthalmic test was applied to the right eye of all animals on the 13th day and to the left on the 43rd day. During this interval one rabbit from each Group B and C had died. On the 48th day all of the animals except two of Group A received a subcutaneous focus of 5 cc. of agar infected with sediment from 5 cc. of culture of Streptococcus V110A. Six fresh rabbits comprising Group D received similarly infected agar foci. All animals were then allowed to go untreated until the 101st day, an interval of 53 days, when their right eyes were retested in the usual manner and their skin reactivity to inoculation with $10^{-4}$ cc. of homologous culture was measured. The comparative intensity of the cutaneous reactions to $10^{-4}$ cc. of culture is shown in Chart 1, and that of the ophthalmic reactions is shown in Table I.

The results clearly indicate that after the animals had been rendered hypersensitive by means of the initial skin inoculation the condition was satisfactorily maintained when an infected focus was present. At that time, however, we did not appreciate the influence of repeated intracutaneous test inoculations with $10^{-4}$ cc. of culture in maintaining

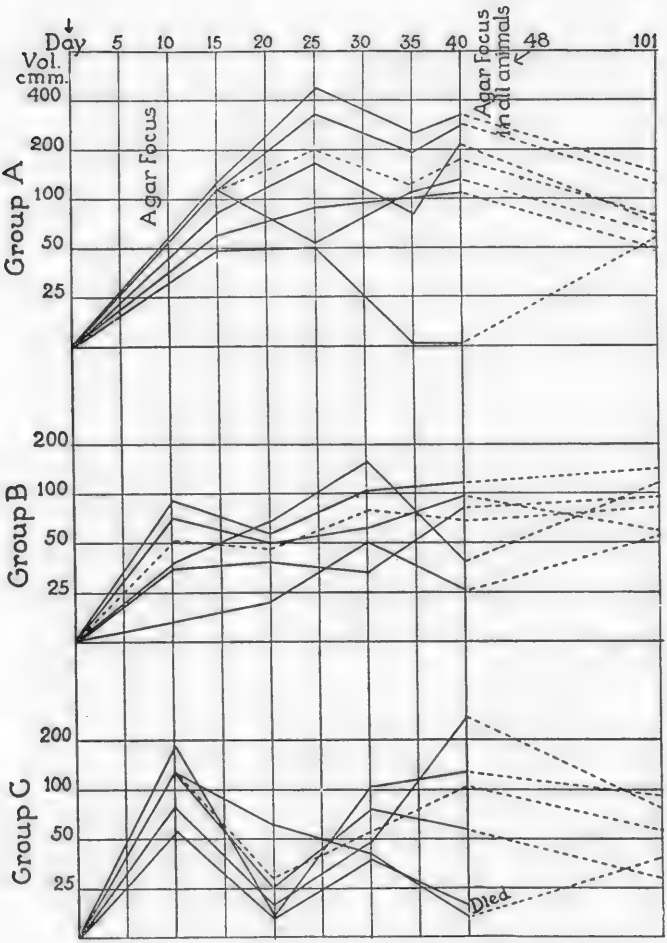


CHART 1. Comparative intensity of cutaneous reaction in rabbits sensitized in different manners.

the hypersensitive state (see Experiment 2). Yet the animals of Groups B and C also received similar small intracutaneous inoculations repeatedly at 10-day intervals concomitantly with the course of

$10^{-1}$ cc. and $10^{-2}$ cc. doses, without developing a comparable degree of hypersensitiveness. Indeed, it seems probable from the appearance of the curves of Group C that the repeated intracutaneous inoculation with these larger doses may have resulted in a temporary diminution of sensitiveness at about the 20th day. This opinion was confirmed by a parallel diminution in the size of the cutaneous lesions resulting from inoculations with $10^{-1}$ cc. and $10^{-2}$ cc. of culture. Other experiments have shown a similar wave-like variation in intensity of reactions in animals receiving repeated intradermal inoculations of large doses of *Streptococcus viridans*.

In this experiment the skin lesions resulting from $10^{-1}$ cc. inocula were usually as large after the third and subsequent inoculations as were those produced by the sediment from 5 cc. at the time of the original inoculation. It is possible that these large cutaneous foci containing much necrotic tissue and many bacteria may have had less sensitizing effect than the smaller areas produced by $10^{-4}$ cc. of culture. This possibility is rendered more probable from comparative consideration of Group B. These animals, although receiving the major portion of the inocula into their lumbar muscles, always showed cutaneous lesions at the site of the needle punctures; lesions that were often of the same size as followed intracutaneous inoculation with $10^{-4}$ cc. of culture. They, therefore, were under the sensitizing influence of two small intracutaneous inocula every 5 days and of three similar additional ones every 10 days. Under these combined muscular and cutaneous lesions, however, the animals seemed to remain at a constantly lower level of sensitivity than existed in Group A, where the repeated sensitizing cutaneous stimuli were very small.

At the end of 6 weeks the average cutaneous and corneal sensitiveness of Group A was distinctly greater than at the end of the 2nd week, and also higher than that of Groups B and C, in which it was relatively the same as in the 2nd week. While on the 13th day none of the Group C animals gave a positive ophthalmic reaction, one did so by the 7th week (see Table I).

The effect of an infected agar focus implanted on the 48th day was to maintain or increase the corneal sensitiveness of nearly all the animals by the 101st day, even though the cutaneous reactivity of Groups A and C had fallen off to a certain degree. It is true that the

TABLE I

*Comparative Intensity of Ophthalmic Reactions in Rabbits Sensitized in Different Manners*

| Group | Rabbit No. | 13th day Rt. eye | | | | 43rd day Left eye | | | | 101st day Rt. eye | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Degree of reaction | Days between inoculation and appearance of: | | | Degree of reaction | Days between inoculation and appearance of: | | | Degree of reaction | Days between inoculation and appearance of: | | |
| | | | Congestion | Veil | Pannus | | Congestion | Veil | Pannus not observed | | Congestion | Veil | Pannus |
| A | R832 | + | 3 | 3 | 5 | +++ | 1 | 1 | | +++ | 1 | 1 | 2 |
| | R833 | + | 2 | 3 | 7 | +++ | 1 | 1 | | ++++ | 1 | 1 | 2 |
| | R834 | − | − | − | − | − | − | − | | ± | 1 | 1 | 0 |
| | R835 | ++ | 1 | 2 | 5 | +++ | 1 | 1 | | +++ | 1 | 1 | 3 |
| | R836 | ± | 3 | 4 | − | + | 2 | 3 | | +++ | 1 | 2 | 5 |
| | R837 | ? | 2 | − | − | +++ | 1 | 1 | | +++ | 1 | 1 | 2 |
| B | R838 | + | + | 1 | − | + | 2 | 2 | | − | − | − | − |
| | R839 | − | − | − | − | − | − | − | | +++ | 1 | 1 | 3 |
| | R840 | − | − | − | − | − | − | − | | +++ | 1 | 2 | 5 |
| | R841 | + | 1 | 1 | 5 | ++ | 1 | 2 | | +++ | 1 | 1 | 6 |
| | R843 | ++ | 1 | 1 | 5 | ± | 1 | 2 | | +++ | 1 | 1 | 3 |
| C | R844 | − | − | − | − | ++ | 2 | 2 | | ++ | 1 | 1 | 6 |
| | R845 | − | − | − | − | − | − | − | | +++ | 1 | 1 | 5 |
| | R847 | − | − | − | − | − | − | − | | ± | 1 | 1 | 0 |
| | R848 | − | − | − | − | − | − | − | | † | | | |
| | R849 | − | − | − | − | − | − | − | | ++ | 1 | 1 | 5 |
| D | R878 | | | | | | | | | − | − | − | − |
| | R879 | | | | | | | | | − | − | − | − |
| | R880 | | | | | | | | | ++ | 7 | 7 | 9 |
| | R881 | | | | | | | | | ± | 8 | 8 | 0 |
| | R882 | | | | | | | | | − | − | − | − |
| | R883 | | | | | | | | | ++ | 4 | 4 | 6 |
| E | Controls | | | | | | | | | | | | |
| | R897 | | | | | | | | | − | − | − | − |
| | R898 | | | | | | | | | + | 10 | 10 | 11 |
| | R899 | | | | | | | | | ++ | 10 | 10 | 12 |
| | R900 | | | | | | | | | − | − | − | − |

† Animal died.

\+ = negative reaction.

± to ++++ = varying intensity of reaction.

right eye, which had been inoculated on the 13th day, was the one tested on the 101st day and the effect of this first inoculation must be considered. But among eight of the surviving animals in which the first inoculation of the right eye did not result in keratitis, the second—on the 101st day—was followed by distinct and intense reactions (see Table I).

These reactions, moreover, appeared promptly as compared with those of Groups D and E. Group D animals had been sensitized 53 days previously by an infected subcutaneous agar focus, without any preliminary cutaneous inoculations. Only one-half of this group developed positive ophthalmic reactions at all, and these were all weaker than in the first three groups, and were also slower in appearing. The members of Group E were introduced for the first time at the end of the experiment, to serve as controls of the activity of the culture at that time; like the animals of the other groups they received intracutaneously $10^{-1}$ cc., $10^{-2}$ cc., and $10^{-4}$ cc. of culture on the day their corneae were inoculated. Two developed secondary cutaneous reactions and shortly afterwards showed weak but distinct ophthalmic reactions. These two animals illustrate well that for the development of keratitis a state of hypersensitiveness is necessary; for even though their eyes were inoculated early, they showed corneal lesions only after the development of this state as indicated by the secondary reactions. The five groups of animals show clearly, as judged by their ophthalmic reactions, how variations in the degree of hypersensitiveness may be conditioned by differences in the method of previous inoculation.

Experiment 1 indicated that in most rabbits a high degree of hypersensitiveness could be obtained in 35 to 40 days by the following method: initial inoculation of several large doses of green streptococci, followed by the introduction of a single infected agar focus, and thereafter by repeated intracutaneous inoculations with $10^{-4}$ cc. of culture at 10-day intervals. In two subsequent experiments carried out in almost the same manner, but without skin testing every 10 days, such a satisfactory degree of hypersensitiveness was not obtained. In fact the proportion of highly sensitive animals was very disappointingly small. The only differences between the two series of experiments consisted in the absence of small intracutaneous

inoculations every 10 days in the second, and in the seasons of the year in which the two were carried out. Variations in sensitizing capacity of culture must also be considered.

Experiment 2 was therefore planned to determine whether one infected subcutaneous agar focus, or multiple intracutaneous foci from repeated single inoculations with $10^{-4}$ cc. of culture every 10 days, or a combination of the two, was most effective in maintaining the hypersensitive state.

*Experiment 2.*—Each of 64 rabbits was inoculated intracutaneously with the sediment of 5 cc., 5 cc., $10^{-1}$ cc., and $10^{-4}$ cc. of blood broth Culture V110A/0/6. On the 8th day they were divided into four groups. Into each animal of Group A (27 rabbits) and Group B (25 rabbits) was placed a subcutaneous focus of 5 cc. of melted agar inoculated with the sediment of 5 cc. of 24-hour blood broth culture. Groups C and D, containing 6 rabbits each, received no agar foci. Groups A and B were larger because it was hoped by production of agar foci to obtain many highly sensitive animals for a subsequent experiment. On the 11th day the right cornea of each rabbit was inoculated in the usual manner. The number and percentage of positive ophthalmic reactions is indicated in Table II. The proportion of animals in Groups A, B, and D, which developed secondary reactions was approximately the same; but Groups A and B had a much higher proportion of positive ophthalmic reactions than did Groups C and D. It seemed, therefore, that the implanting of agar foci at the time when the animals were becoming hypersensitive increased the sensitiveness of the eyes; this is a confirmation of similar observations in Experiment 1. The animals of Groups A and C were inoculated intracutaneously with $10^{-4}$ cc. of homologous culture on the 11th, 21st and 31st days. On the 42nd day the left eye of all the rabbits was tested, and all animals received $10^{-4}$ cc. of culture intracutaneously. The volumes of the resulting skin lesions were computed from daily measurements; the average for each group is given in Table II. Five days later the cutaneous reactivity of all the rabbits was again tested with $10^{-4}$ cc. of each of two cultures, one of which had been grown from an old blood broth stock preserved in the ice-box, while the other had been inoculated from a stock recently derived from the original frozen and dried material. The lesions induced by these two cultures were quite comparable in size; the average volume is recorded in Table II.

At the end of 6 weeks each of the four groups had been subjected to different sensitizing conditions. In so far as the secondary reaction is an indicator of sensitivity all groups on the 11th day seemed to be about equally hypersensitive; but the eye test showed that Group D was less so than the others. While at this early period only one animal of Group C had developed a marked, and one a slight, keratitis,

three subsequent intracutaneous injections of $10^{-4}$ cc. of culture at 10-day intervals apparently so increased the corneal hypersensitiveness of animals of this group that when tested on the 42nd day, three developed strongly positive ophthalmic reactions. In Group A, on the other hand, the degree of corneal sensitiveness was found to have decreased, even though the animals had received the same intracutaneous treatment as those of Group C. But by contrast the

TABLE II

*Summary of Effect of Agar Foci and Repeated Intracutaneous Inoculation with $10^{-4}$ cc. of Culture on Maintaining the Hypersensitive State in Sensitized Rabbits*

| Group | No. of animals | 1st day | 8th day | 11th day | | | | 11th day 21st " 31st " | 42nd day | | | 47th day |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Secondary reaction | | Ophthalmic reaction | | | Ophthalmic reaction | | | |
| | | Sediment of 5 cc., 5 cc., $10^{-1}$ cc. and $10^{-4}$ cc. intracutaneously | Agar focus inoculated with sediment of 5 cc. | + | − | + | − | Intracutaneous reinoculation $10^{-4}$ cc. | + | − | Intracutaneous inoculation $10^{-4}$ cc. Average vol. in cmm. | Intracutaneous inoculation $10^{-4}$ cc. Average vol. in cmm. |
| A | 27 | + | + | 21<br>78% | 6 | 19<br>76% | 8 | Yes | 12<br>44% | 15 | 75 | 102 |
| B | 25 | + | + | 17<br>68% | 8 | 18<br>72% | 7 | No | 6<br>24% | 19 | 52 | 64 |
| C | 6 | + | 0 | 3<br>50% | 3 | 2<br>33% | 4 | Yes | 3<br>50% | 3 | 77 | 133 |
| D | 6 | + | 0 | 4<br>67% | 2 | 0<br>0% | 6 | No | 0<br>0% | 6 | 34 | 62 |

continual sensitizing effect of these small repeated inocula is illustrated in Groups B and D, in which they had been omitted: Group B on the 42nd day gave approximately one-third the number of positive eye reactions that it did on the 11th day, and Group D failed entirely to develop sufficient hypersensitiveness for demonstration by the ophthalmic test. The comparative intensities of the skin reactions in the various groups on the 42nd and 47th days also showed that the repeated production of cutaneous foci with only $10^{-4}$ cc. of culture

was more favorable for the continuation of the hypersensitive state than was the production of a single subcutaneous agar focus. The stronger reactions of all of the animals on the 47th day as compared with the 42nd indicate either that the culture used for testing was more potent or that the animals were more reactive. As the two different cultures used for testing on the 47th day gave reactions of approximately the same intensity, it is probable that the cutaneous sensitivity of all of the rabbits had been elevated by the small cutaneous inoculation 5 days previously. The experiment in all of its phases indicates clearly that after animals are made hypersensitive, this state can be continued or increased by cutaneous foci induced by doses of streptococci which are incapable of stimulating macroscopically visible lesions in normal animals.

In practically all previous experiments it had been assumed that a first inoculation of the sediment of 5 cc. of culture was necessary for the development of the hypersensitive state; in other words, that a large amount of bacterial substance was the important factor in giving the initial impulse. But control groups in a number of experiments, such as Group E in Experiment 1, had shown that distinct hypersensitiveness might follow the production of three lesions with $10^{-1}$ cc., $10^{-2}$ cc. and $10^{-4}$ cc. of culture respectively. Moreover, in a comparison of the sensitizing capacities of a large number of strains of indifferent streptococci (3) it was found that nine small lesions often led to a marked degree of hypersensitiveness. It seemed advisable, therefore, to determine whether the total dosage of streptococci injected intracutaneously or the number of lesions produced was the more important in inducing a condition of hypersensitiveness, provided the lesions were all made at one time.

*Experiment 3.*—Five groups of 4 rabbits each were inoculated intracutaneously in a varying number of places with different doses of indifferent Streptococcus Q186A/0/6, as shown in Table III. The number of lesions varied between 1 and 11 and the total dosage was the centrifugate of either 0.222 cc., 5.0 cc., or 5.222 cc. of a 20-hour blood broth culture. All of these lesions were measured daily to detect secondary reactions; and on the 14th day the comparative hypersensitiveness of the animals was tested with intracutaneous inocula of $10^{-2}$ cc., $10^{-4}$ cc., and $10^{-6}$ cc. of a homologous culture, and the ophthalmic test was applied to each. A new group (F) of normal controls was introduced at this time to test the activity of the culture.

Two rabbits of Group A sickened of *B. lepisepticum* infection; hence comparison with the others is somewhat vitiated. But the two survivors developed secondary reactions, and showed lesions on reinfection comparable with those of the other groups. Moreover, the results of the rest of the experiment do not indicate that these Group A animals would necessarily have been less sensitive than any of the others. Group E, the members of which received only a single injection of 5 cc. of centrifugate, was the most poorly sensitized. Group C, on the

TABLE III

*Comparative Sensitizing Effect of Different Doses of Streptococci Distributed in a Varying Number of Lesions*

| Group | Amount of inoculum | | | | | Each animal | | Total with secondary reaction | Test 14th day | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 cc. | 1 cc. | $10^{-1}$ cc. | $10^{-2}$ cc. | $10^{-3}$ cc. | Total lesions | Total dose | | Positive ophthalmic reaction | Average maximum volume retest on 14th day | | No. of animals showing reaction with |
| | | | | | | | | | | $10^{-3}$ cc. | $10^{-4}$ cc. | $10^{-4}$ cc. |
| A No. of lesions | 1 | ... | 2 | 2 | 2 | 7 | 5.222 | 2* | 1 | 367** | 96** | 2 |
| B induced by | ... | 5 | 2 | 2 | 2 | 11 | 5.222 | 3 | 1 | 375 | 79 | 2 |
| C inocula of | ... | ... | 2 | 2 | 2 | 6 | 0.222 | 3 | 2 | 325 | 55 | 3 |
| D respective | ... | 5 | ... | ... | ... | 5 | 5.0 | 3 | 2 | 532 | 101 | 4 |
| E size | 1 | ... | ... | ... | ... | 1 | 5.0 | 1 | 1 | 387 | 36 | 2 |
| F | | | | | | | | | | | | |
| Controls | ... | ... | ... | ... | ... | ... | ... | ... | 0 | 85 | 0 | 0 |

* Only 2 rabbits of Group A survived until the 14th day.

** Figure indicates average for the group.

other hand, which received approximately only 1/20th of the dose used in the other animals but divided amongst six areas, developed hypersensitiveness in as many members as either Group B or D. The latter group, however, showed the largest lesions following reinoculation.

The culture used in this experiment was capable of producing in normal animals lesions about 1 cm. in diameter with doses of $10^{-3}$ cc., but no macroscopic lesions with $10^{-4}$ cc. In other experiments it has been observed that unless a particular strain could evoke fair

sized lesions with doses of at least $10^{-1}$ cc. or $10^{-2}$ cc. it was practically useless in eliciting secondary reactions or in inducing a state of hypersensitiveness of sufficient intensity to be detected by the ophthalmic test. One may conclude, therefore, that if with any suitable strain lesions of about 10 mm. diameter are produced by relatively small doses, a number of these small lesions will more readily induce a hypersensitive state in rabbits than will a single lesion made by injecting a much larger quantity.

The demonstration that intracutaneous injection of non-hemolytic streptococci at 10-day intervals, in doses 1/10th the size necessary to produce minimal macroscopic lesions in normal animals, either maintained or enhanced the hyperergic state in rabbits already somewhat hypersensitive, made it appear that possibly it was the amount of tissue injured in a peculiar manner rather than the quantity of bacterial substance which furnished the direct stimulus for hypersensitization.

The time (30 to 60 days) required by this method to render the animals sufficiently hypersensitive for further experimental procedures made it desirable to determine whether the same principle might not be applied to secure a more rapid sensitization of the animals.

*Experiment 4.*—The microorganism, P64, used in this experiment, was a green-forming streptococcus, which in doses of $10^{-2}$ cc. of blood broth culture induced in normal rabbits lesions of 10 to 15 mm. in diameter and about 1 mm. in height; these usually persisted for 1 to 3 days. Macroscopic lesions were usually not visible at the site of $10^{-3}$ cc. inoculation; but at the sites of injection of 1 cc. and $10^{-1}$ cc. there occurred long tracking zones of redness and edema which gradually disappeared, and left areas in which secondary reactions were most likely to occur. Each rabbit of two groups was inoculated intracutaneously with 1 cc., $10^{-1}$ cc., $10^{-2}$ cc., and $10^{-3}$ cc. of a 24-hour blood broth culture. Group A animals were simply observed in the usual manner; but those of Group B received during the next 2 weeks 11 injections each of $10^{-3}$ cc. of similar cultures; these inoculations were made daily with the exception of the 6th and 13th days; and each resulting lesion was measured daily until it diminished in size. On the 15th day the ophthalmic test was applied to the left eyes, and on the 22nd day all of the rabbits were inoculated intravenously with the sediment of equivalent amounts per kilo body weight of 24-hour blood broth culture. The results are summarized in Table IV.

It is obvious that daily inoculations for 2 weeks induced distinct hypersensitiveness in all of the Group B animals, while those of Group A receiving merely the original inocula failed to become sufficiently sensitive to respond to any of the three tests used to detect this state. It is not to be expected that such a clear cut difference between the two groups would always appear, for with other strains a considerable degree of hypersensitiveness has often been induced in some animals by a single series of inoculations similar to or smaller than that used in Group A. With this strain, however, we were fortunate in obtaining conditions favorable for the illustration of the advantage of the added daily inoculation of small doses.

TABLE IV

*Comparative Sensitizing Effect of Initial Inoculum and a Similar Inoculum Followed by Daily Inoculation*

| Group | Rabbit No. | Secondary reaction | | Reaction from $10^{-3}$ cc. inoculation | | 15th day ophthalmic reaction | 22nd day lethal reaction |
|---|---|---|---|---|---|---|---|
| | | $10^{-0}$ cc. | $10^{-1}$ cc. | Begun | Maximum | | |
| A | R2126 | 0 | 0 | ... | ... | 0 | 0 |
| | R2127 | 0 | 0 | ... | ... | 0 | 0 |
| | R2129 | 0 | 0 | ... | ... | 0 | 0 |
| B | R2130 | ± 10th day | ++++ 12th day | 4th day | 10th day | ++ | + |
| | R2131 | + 12th day | +++ 7th day | 3rd day | 9th day | ++ | + |
| | R2132 | ++++ 12th day | ++++ 15th day | 4th day | 10th day | ? | S* |

* Sick, but recovered.

Another point brought out in this experiment was the early onset of hypersensitiveness, detected by measuring the $10^{-3}$ cc. lesions in Group B (see Chart 2). Whereas the first two or three areas inoculated with this dose showed little or no gross evidence of infection, those following the third or fourth inoculation developed distinct maculopapules, and each subsequent injection induced a lesion of greater volume than the previous one. The maximum reaction occurred in areas inoculated on the 9th or 10th days, or about the time of appearance of the secondary reactions at the sites of the original larger inoculations. Then the intensity of the reactions following

reinoculation seemed to diminish somewhat, a suggestion that an "immune state" was being superimposed on the pre-existing hyperergic condition. Similar diminution in reactivity has been observed by Julianelle (4) in sensitizing rabbits with pneumococcus vaccines.

Still another advantage is suggested for this mode of sensitization. The recording of the amount of reaction occurring at the site of each reinoculation discloses, by the end of the first week, the probable capacity of the animal to develop a satisfactory state of hypersensitive-

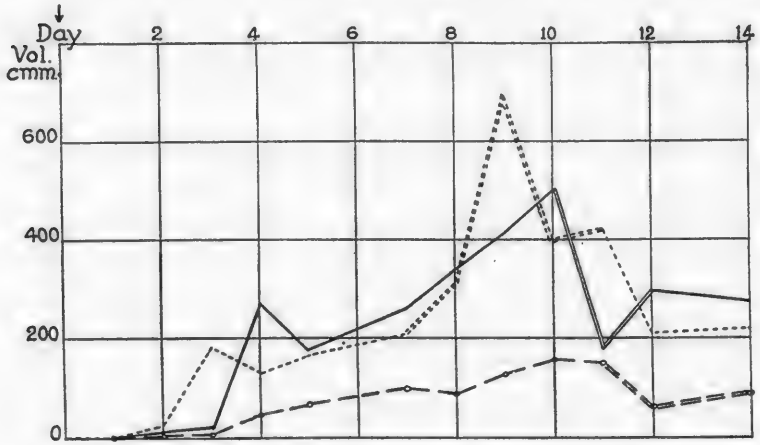


CHART 2. Volume of lesions resulting from daily inoculation with $10^{-3}$ cc. of Streptococcus P64.

Double lines indicate time of appearance of secondary reaction at site of original inoculations.

ness. This point is brought out by comparing Rabbit R2132 with the other two of the group (see Chart 2). The degree of reaction was slow in increasing, and was always small as compared with that of the other two animals; the secondary reaction was correspondingly slow in developing; the ophthalmic reaction was equivocal; and intravenous inoculation merely caused the animal to sicken without leading to death. In subsequently applying this mode of sensitization we have been able to determine within 7 to 10 days that certain animals were showing the cachectic type of response or failing to become satis-

factorily hypersensitive, and thus to eliminate them from any series to be used for comparative studies.

From Experiment 4 it was not clear whether daily inoculations with small doses of streptococci for 2 weeks without the initial larger inocula might not have been as efficacious as the two combined; hence additional experiments were performed to answer this question.

*Experiment 5.*—Streptococcus V110A was used as the sensitizing strain. The animals of Groups A and B were inoculated in a manner similar to those in Experiment 4; but those of Group C received only daily inoculations of $10^{-3}$ cc. amounts over a period of 2 weeks. Ophthalmic tests did not reveal sharply defined differences amongst the various groups; but in this experiment, as well as in Experiment 4, the findings at autopsy after comparable intervals following lethal tests showed the most severe and widespread lesions in animals of Group B, which had received daily small inoculations in addition to an initial series of larger doses; the members of Group C, which had been sensitized with daily inoculations alone, showed somewhat more extensive lesions than those of Group A which had received simply the initial inoculations.

Comparison of the average volumes of the reactions from repeated $10^{-3}$ cc. inocula showed that in the 2-week period the animals of Group B had somewhat larger lesions; hence one may conclude that in this period the strong initial stimulus combined with daily inoculation was more efficacious than the daily small inoculations alone.

*Experiment 6.*—In Experiment 6 the reactions to daily inoculation of $10^{-3}$ cc. of blood broth culture of *Streptococcus viridans* V110A were followed for 4 weeks. In Group B initial inocula of 1 cc., $10^{-1}$ cc., and $10^{-2}$ cc. were given; in Group C these were withheld, while in both groups single daily injections of $10^{-3}$ cc. were made. The average of the two groups is shown in Chart 3.

In all animals a wave-like rise and fall of cutaneous reactivity was observed. The curve rose most rapidly in the first 10 or 12 days; then there was a fall followed by a second peak about the 20th day, following which a second fall occurred.[1] All animals of Group B then showed a stationary or falling trend; in one this was very marked as though the animal were becoming "immune." It was impractical to carry the experiment further because most of the good available

[1] A somewhat similar wave-like variation in reactivity to repeated intracutaneous inoculation is recorded by Krause and Willis in tuberculous guinea pigs (*Am. J. Tuberculosis*, 1926, **14**, 316).

skin had been utilized. This experiment demonstrates, however, that a high degree of sensitivity can be attained by repeated daily inocula of relatively small doses of non-hemolytic streptococci, when carried out over a prolonged period.

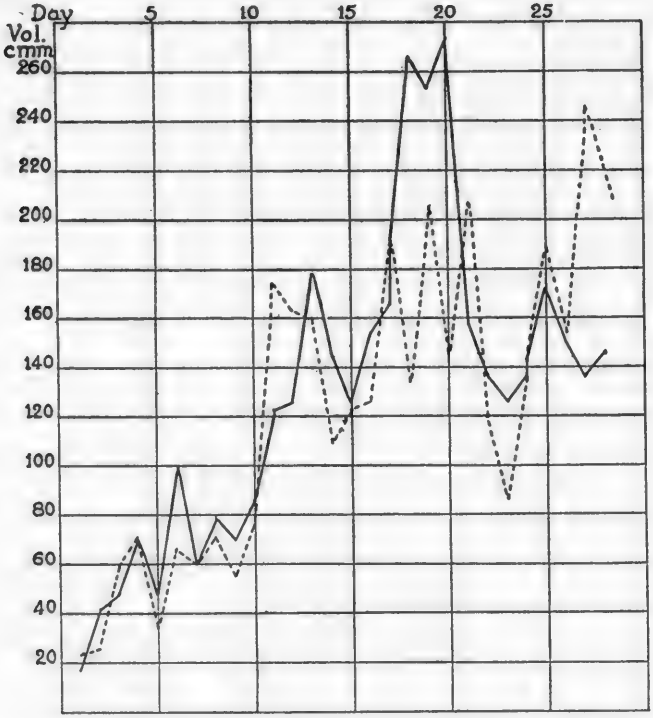


CHART 3. Comparative intensity of reactions at site of daily inoculations of $10^{-3}$ cc. of Streptococcus V110A.

Solid line, Group B received original inoculation of 1, $10^{-1}$ and $10^{-2}$ cc. followed by daily inoculation of $10^{-3}$ cc. Broken line, Group C received daily inoculation of $10^{-3}$ cc. only.

The tendency for the lesions to be less marked during certain periods in animals receiving daily inocula suggested that possibly the hypersensitive state might be temporarily depressed as a result of too

frequent inoculation; and in addition Experiment 2 suggested that after a certain degree of hypersensitivity had been attained, less frequent inoculations would serve to maintain or heighten this condition. Experiment 7, therefore, combined certain features of the two methods.

TABLE V

*Comparison of Degree of Hypersensitiveness of Corneae after 2 and 9 Weeks*

| No. animals | Strain | After 2 weeks' sensitization. Ophthalmic reaction | | | After 7 weeks' additional sensitization $10^{-3}$ cc. and $10^{-4}$ cc. each week | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Ophthalmic reaction | | | Lethal test. No. died |
| | | − | ± | + to ++++ | − | ± | + to ++++ | |
| 3 | Q155 | ... | 1 | 2 | ... | 1 | 2 | 2* |
| 3 | Q167B | 3 | ... | ... | 1 | ... | 2 | 3 |
| 2 | R1 | 2 | ... | ... | 1 | ... | 1 | 2 |
| 4 | R9 | ... | 2 | 2 | ... | ... | 4 | 4 |
| 2 | RF156 | ... | ... | 2 | ... | ... | 2 | 2 |
| 3 | RF162B | 3 | ... | ... | 2 | ... | 1 | 0 |
| 3 | A49 | 1 | 2 | ... | ... | ... | 3 | 3 |
| Total, 20 | | 9 | 5 | 6 | 4 | 1 | 15 | 16 |
| Per cent..... | | 45 | 25 | 30 | 20 | 5 | 75 | 80 |

* The other animal of the group rendered very sick, but recovered. Autopsy findings showed typical changes.

*Experiment 7.*—Seven different strains of streptococci, six indifferent and one green-forming, were employed; each rabbit received but a single strain, with a distribution of strains among groups as shown in Table V. The inoculation on the first day consisted of 1 cc., $10^{-1}$ cc., $10^{-2}$ cc., and $10^{-3}$ cc. of blood broth culture; thereafter daily inocula of $10^{-3}$ cc. were given for 2 weeks, at the end of which time the right corneae were inoculated with homologous streptococci. For the next 7 weeks each animal received $10^{-3}$ cc., and $10^{-4}$ cc. of homologous culture every 7 days. The left eyes were then tested; and 1 week later all animals were inoculated intravenously with the usual lethal test dose of the homologous strain. The results are summarized in Table V.

It is evident that the 7 weeks' additional treatment greatly increased the hypersensitiveness of the entire group. While somewhat more than one-half of the animals showed some degree of corneal sensitivity

at the end of 2 weeks, in one-fourth it was only slight. Seven weeks later, however, the number of strong ophthalmic reactions had more than doubled. The lethal test, moreover, indicated that all of the animals except those inoculated with Strain RF162B had been rendered very hypersensitive by the prolonged treatment; this was a much larger number of positive lethal reactions than would have been expected at the end of 2 weeks in a group of animals showing so few strongly positive ophthalmic reactions. In fact, if the animals inoculated with Strain RF162B be deleted from the experiment, and the one severely but not fatally shocked animal of the Q155 group be included, it can be said that all of the rabbits inoculated with six of the strains showed at the end of 9 weeks marked general hypersensitivity. The RF162B group illustrates the necessity of employing suitable strains in order to obtain satisfactory sensitivity, for although these animals were treated in identically the same manner as the others, their skin reactions were always poor and none of them was rendered sufficiently sensitive to sicken after intravenous inoculation.

## DISCUSSION

Several interesting points have emerged during the progress of this work. At the beginning it was thought that the degree of hypersensitiveness would be more or less proportional to the size of the initial dose. This was to be expected from the work of Roemer (5) Krause (6) and others on hypersensitiveness in experimental tuberculosis, and of Bloch (7) and his co-workers in trichophyton infections. But it soon became evident that, within certain limits, the initial production of multiple lesions with smaller doses of non-hemolytic streptococci was more likely to lead to marked hypersensitivity than that which followed a single lesion induced by larger amounts; furthermore, the most satisfactory hypersensitiveness followed repeated inoculations with small doses of the microorganisms. In a number of instances these doses were too small to induce macroscopic lesions in normal animals.

The results of infection of animals with single inocula of non-hemolytic streptococci and with tubercle bacilli are not exactly comparable. In the first instance, as the microorganisms are so quickly killed they do not multiply long at the site of inoculation and are dis-

seminated to other areas for only a short time. In the second case they grow and are carried to distant organs, where they again increase and produce lesions; hence in the first instance relatively few local lesions are produced, while in the second case multiple and disseminated injuries occur. Repeated small inoculations with streptococci, therefore, probably reproduce more nearly the conditions following a single inoculation with tubercle bacilli. This view point is substantiated by the results of implanting agar foci infected with streptococci. Such foci harbor living streptococci for a period of from 3 to 4 weeks, but even after becoming sterile they presumably contain disintegrated bacterial sensitizing products for long periods. Nevertheless these foci have not proven as efficacious in increasing the sensitive state as have repeated inocula of small size. This suggests that the kind of lesion rather than the amount of bacterial substance is the important factor. Indeed, lesion—tubercle production—is the *sine qua non* for the development of tuberculin allergy; and it is highly suggestive that Zinsser and Mueller (8) found that the production of multiple skin tuberculous lesions in rabbits yielded the best conditions for passive transfer of tuberculin allergy. They stress the rôle of the focus in the induction of "allergy;" but later Zinsser (9) thought that the peculiar manner in which the bacteria are disintegrated was the important factor. Whatever may be formed from the bacteria in a focus, our experiments indicate that only exceedingly small amounts of the substance are necessary to increase the hypersensitive condition, once the initial stimulus has been provided for the development of that state.

Experience with various infections indicates that there are two types of reaction towards reinfection with microorganisms—that of hypersensitivity, and that of immunity. That the two are often intimately linked is indicated by the opinions of most workers in this field. But there is a growing tendency to regard the two processes as not necessarily interdependent (Boquet and Nègre (10), Swift and Derick (1, 2), Rich (11) ). One problem of experimental investigation is to determine the best manner of imposing these two types of reaction, each independently of the other, on different animals, in order to study their respective rôles in the general subject of resistance. Julianelle (4) noted that rabbits sensitized by intracutaneous injection of vaccines of pneumococci showed increasing lesions up to a certain time;

there was then a decrease which was associated with the appearance of immune bodies in the serum. Panton and Valentine (12) observed that rabbits receiving repeated inoculations with living staphylococci eventually showed decreasing reactions to large inocula and increasing reactions to small ones, another example of concomitant hypersensitiveness and immunity. Both groups of observers employed relatively large inocula; although the latter stated that hypersensitiveness could be induced by small doses.

From a theoretical standpoint it might be expected that when relatively large amounts of bacterial substances are acting within an animal's body there is more possibility of wide distribution and accompanying immunity. With small amounts, there is simple focal lesion-production, and attending hypersensitivity. Our experiments indicate that repeated small focal lesions usually lead to increasing general tissue hypersensitivity without local evidence of immune tissue response. Unfortunately the relative avirulence of the non-hemolytic streptococci makes it impossible to test the general immunity of rabbits so sensitized; but we have observed that rabbits may be highly sensitized and show little or no serum agglutinin against the sensitizing microorganism, and little precipitin against its nucleoprotein. Contrasted with this may be cited the observations of Fleischer and Meyer (13) that following intraperitoneal injections of vaccines prepared from *B. abortus* or *B. typhosus*, guinea pigs would yield high agglutinins against the homologous bacterium without giving abortin or typhoidin reactions.

The influence of chronicity of infection as a sensitizing factor is indicated by a number of our experiments; and again let it be emphasized that the dose of bacteria sufficient to increase the hypersensitive state was too small to induce macroscopic lesions in normal animals. Thus in the hypersensitive animal there are set up conditions favorable for the augmentation of this state in the presence of a minimum dose of antigen. Klinge (14) has recently emphasized the importance of chronicity in the production of certain peculiar reactions to coagulable protein; while the observations of Dienes (15) suggest that the local condition of the tissue into which a coagulable protein is introduced may also determine the subsequent type of reaction to that protein. Both factors doubtless have important influences, but in order to

evaluate them the importance of dosage of antigen must be considered more carefully than in the past. In experimentally inciting the hypersensitive state (tuberculin-like allergy or hypersensitiveness of infection) large doses of bacteria have usually been employed; but in natural infections characterized by so-called "allergy"—better "hyperergy"—the infecting dose of microorganisms is usually small, and presumably the inciting of new lesions at a distance from the initial lesions is usually effected by relatively few germs which doubtless multiply to a certain extent at the site of metastatic implantation. But in syphilis, which is a classical example of changing "allergic" reactions, it is noteworthy that the most destructive lesion, the gumma, usually arises only after the spirochetes have been acting in the body for a number of years; and at times antecedent evidences of infection—in other words, lesions—may have been so small as to escape detection. Other clinical examples of "allergic alteration" of tissues might be cited to show that in nature the hypersensitive state is induced or maintained by small lesions existing or reproduced over considerable periods of time.

## SUMMARY

The most satisfactory method thus far found for the induction and maintenance of a high degree of hypersensitiveness—"allergy," "hyperergy"—against non-hemolytic streptococci consists in the repeated production of small focal lesions with minimal doses of bacteria.

After a preliminary sensitizing period of about 2 weeks' duration with either large initial, or small multiple daily inoculations, the later foci need be produced only at 7 to 10 day intervals.

Chronicity of low-grade infection appears to be an important factor in the attainment of a high degree of hypersensitiveness.

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# STUDIES IN THE BLOOD CYTOLOGY OF THE RABBIT

## II. Consecutive Erythrocyte and Hemoglobin Observations on Groups of Normal Rabbits

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In the first paper of this series dealing with the blood cytology of the rabbit, the results of 1110 blood counts on 174 normal male rabbits were considered primarily from the standpoint of the numerical variations of the various classes of cells and of the hemoglobin content in individual determinations (1). Other phases of this investigation have included repeated observations on groups of normal and diseased rabbits for extended periods of time. In the case of normal animals, the study was undertaken chiefly for two reasons. Firstly, information was desired concerning the character of the spontaneous fluctuations in the numbers of blood cells under prolonged conditions of undisturbed indoor life with the idea that eventually some relationship might be found between these variations and certain environmental conditions, as was demonstrated with the weights of organs and the amount and degree of change of the total sunlight (2). Secondly, it was considered essential to have information of this nature as a background for whatever changes might be found in association with certain subacute or chronic experimental conditions, as for example, *Treponema pallidum* infection. Five groups of rabbits, 4 of 10 and 1 of 5 animals, were followed 35, 13, 8, 29, and 26 weeks respectively, covering a period of 20 months from October, 1927 to July, 1929. The group examinations numbered 111, in which 973 individual counts are represented.

The results of this study which have been analyzed statistically, will be reported in the present and subsequent papers. For the time being, the principal interest centers in the consecutive mean values of the various classes of cells considered from the standpoint of the trends

of numerical levels shown by each animal group. In the present paper, the erythrocytes and the hemoglobin content are considered; other papers will deal with the total white cells and their division into granular and non-granular cells (3), with the neutrophiles (pseudo-eosinophiles), the basophiles, and the eosinophiles (4), and with the lymphocytes and the monocytes (5). The results will ultimately be analyzed from the standpoint of the relationships of the various classes of cells as shown by their correlation coefficients and in addition, they will be referred to in connection with the relation of the pre-inoculation blood picture to the reaction of the host to disease agents.

## *Materials and Methods*

The rabbits employed were representative of those used in other experiments carried out in this laboratory; all were male animals approximately 6 to 8 months old. The ordinary brown and grey type predominated but the type described as the Flemish cross or mixture was also represented and there were a few black animals. Each rabbit was separately caged in a well lighted (sunlight), well ventilated room; the diet throughout the period of observation consisted of hay, oats, and cabbage.

The results reported are based upon 111 group examinations carried out at weekly intervals; in a few instances, the counts were made at shorter or longer intervals. The total number of individual blood counts was 973. The observations were made on 45 rabbits comprising 4 groups of 10 and 1 group of 5 animals:

| Group | Number of rabbits | Number of examinations | Number of counts | First count | Last count |
|---|---|---|---|---|---|
| I | 10 | 35 | 350 | Oct. 24, 1927 | June 20, 1928 |
| II | 10 | 13 | 130 | Mar. 29, 1928 | June 19, 1928 |
| III | 10 | 8 | 80 | Sept. 20, 1928 | Nov. 22, 1928 |
| IV | 10 | 29 | 283 | Nov. 27, 1928 | June 18, 1929 |
| V | 5 | 26 | 130 | Dec. 29, 1928 | June 21, 1929 |
| | 45 | 111 | 973 | | |

A uniform routine with respect to the time and the method of collection and examination of the blood was followed as previously described (1). In the case of Group I, half of the animals were examined on one day and half on the following day of each week; all animals in each of the other groups were examined on the same day. The differential white blood determinations were made with the supravital neutral red technic; 100 cells were counted in each specimen.

In the case of Group I, additional specimens (10 cc.) of blood were taken for

chemical examination. During the first 2 months of the experiment, this procedure was carried out at weekly intervals; from January to April, the period was extended to 2 weeks, and the last bleeding on May 17 was made 4 weeks after the preceding one. These bleedings always followed the blood counts by 1 or 2 days in order that as long a time as possible would elapse before the next blood count.

In the analysis of results, absolute numbers of cells per cubic millimeter of blood have been used. The curves obtained by plotting the actual group means were smoothed by the formula $\frac{a + 2b + c}{4}$, the initial value of each curve being represented by $\frac{2b + c}{3}$ and the final value by $\frac{a + 2b}{3}$. The coefficients of variation of the means have also been smoothed by this formula. The smoothed mean values have been compared, in the form of percentage deviations, with the mean numbers of cells found in 1110 normal blood counts (1). For convenience, certain figures used in this comparison as so-called standard values differ slightly from the actual results obtained, as shown in the following tabulation:

| | Actual values | Values used |
|---|---|---|
| | *per cmm.* | *per cmm.* |
| Red blood cells | 5,198,000 | 5,200,000 |
| Hemoglobin | 63% | 63% |
| White blood cells | 9562 | 9560 |
| Neutrophiles | 4341 | 4340 |
| Basophiles | 950 | 950 |
| Eosinophiles | 214 | 215 |
| Lymphocytes | 3045 | 3050 |
| Monocytes | 1000 | 1000 |
| Total granular cells | 5504 | 5505 |
| Total non-granular cells | 4045 | 4050 |

It should be pointed out that the results of the first 3 groups of animals here reported representing 560 counts, appear among the 1110 counts of the above tabulation; the 413 counts of the 4th and 5th groups, on the other hand, are not included in the large group.

The figures as given include all data. No count has been omitted because of the occurrence of such conditions as snuffles or ear canker in certain animals at some time during the period of observation, but these instances will be referred to in the discussion of the results.

## RESULTS

The results pertaining to the consecutive weekly erythrocyte counts and the hemoglobin contents obtained in 5 groups of normal rabbits

TABLE I

*Group I—10 Rabbits. Consecutive Values for Erythrocytes and Hemoglobin*

| Date | Red blood cells (000 omitted) | | | Hemoglobin | | |
|---|---|---|---|---|---|---|
| | Mean values | Standard deviation | Coefficient of variation | Mean values | Standard deviation | Coefficient of variation |
| *1927–28* | *per cmm.* | *per cmm.* | *per cent* | *per cent* | *per cent* | *per cent* |
| Oct. 24* | 5420 ± 192 | 901 | 16.62 | 54 ± 2.1 | 10 | 18.52 |
| Nov. 1 | 5539 ± 42 | 199 | 3.59 | 49† ± 0.4 | 2 | 4.08 |
| Nov. 8** | 4991 ± 79 | 372 | 7.45 | 55 ± 1.4 | 7 | 12.73 |
| Nov. 15 | 5039 ± 92 | 433 | 8.59 | 59 ± 1.3 | 6 | 10.17 |
| Nov. 22 | 5204 ± 130 | 611 | 11.74 | 52 ± 1.3 | 6 | 11.54 |
| Nov. 29 | 5351 ± 76 | 355 | 6.63 | 63 ± 1.3 | 6 | 9.52 |
| Dec. 6 | 5323 ± 229 | 576 | 10.82 | 63 ± 2.1 | 10 | 15.87 |
| Dec. 13 | 5075 ± 143 | 670 | 13.20 | 60 ± 1.4 | 7 | 11.67 |
| Dec. 20 | 5032 ± 170 | 799 | 15.88 | 60 ± 1.4 | 7 | 11.67 |
| Dec. 27 | 5296 ± 118 | 555 | 10.48 | 59 ± 1.4 | 7 | 11.86 |
| Jan. 3 | 5286 ± 129 | 605 | 11.45 | 57 ± 2.3 | 11 | 19.30 |
| Jan. 10 | 4889 ± 115 | 540 | 11.05 | 64 ± 1.4 | 7 | 10.94 |
| Jan. 17 | 4859 ± 182 | 851 | 11.81 | 61 ± 1.4 | 7 | 11.48 |
| Jan. 24 | 5513 ± 126 | 591 | 10.72 | 67 ± 1.4 | 7 | 10.45 |
| Jan. 31 | 5399 ± 176 | 825 | 15.28 | 64 ± 1.7 | 8 | 12.50 |
| Feb. 7 | 5474 ± 145 | 679 | 12.40 | 68 ± 1.3 | 6 | 8.82 |
| Feb. 14 | 5204 ± 153 | 719 | 13.82 | 68 ± 1.3 | 6 | 8.82 |
| Feb. 21 | 5336 ± 156 | 730 | 13.68 | 71 ± 1.3 | 6 | 8.45 |
| Feb. 28 | 5312 ± 178 | 834 | 15.70 | 74 ± 1.0 | 5 | 6.76 |
| Mar. 6 | 5158 ± 101 | 475 | 9.21 | 69 ± 1.7 | 7 | 10.14 |
| Mar. 13 | 5451 ± 155 | 725 | 13.30 | 63 ± 1.3 | 6 | 9.52 |
| Mar. 20 | 5024 ± 116 | 544 | 10.83 | 67 ± 1.3 | 6 | 8.96 |
| Mar. 27 | 5229 ± 200 | 936 | 17.90 | 72 ± 2.1 | 10 | 13.89 |
| Apr. 3 | 4838 ± 134 | 628 | 12.98 | 66 ± 1.9 | 9 | 13.64 |
| Apr. 10 | 5273 ± 96 | 451 | 8.55 | 68 ± 2.1 | 10 | 14.71 |
| Apr. 17 | 5455 ± 83 | 388 | 7.11 | 64 ± 1.0 | 5 | 7.81 |
| Apr. 24 | 5050 ± 180 | 844 | 16.71 | 58 ± 1.9 | 9 | 15.52 |
| May 1 | 5310 ± 143 | 696 | 13.11 | 64 ± 1.4 | 7 | 10.94 |
| May 8 | 5560 ± 117 | 549 | 9.87 | 66 ± 2.3 | 11 | 16.67 |
| May 15 | 5188 ± 110 | 515 | 9.93 | 66 ± 1.7 | 8 | 12.12 |
| May 22 | 5335 ± 205 | 962 | 18.03 | 66 ± 2.1 | 10 | 15.15 |
| May 29 | 5597 ± 162 | 761 | 13.60 | 64 ± 1.0 | 8 | 12.50 |
| June 5 | 6080 ± 205 | 959 | 15.77 | 66 ± 1.3 | 6 | 9.09 |
| June 12 | 5730 ± 110 | 518 | 9.04 | 68 ± 1.9 | 9 | 13.24 |
| June 19 | 5779 ± 111 | 522 | 9.03 | 69 ± 0.8 | 4 | 5.80 |
| Mean | 5303 ± 30 | 263 | 4.96 | 64 ± 0.7 | 6 | 8.59 |
| Minimum | 4838 | | | 52 | | |
| Maximum | 6080 | | | 74 | | |

* October 24 and 26.

** November 4 and 9.

† Mean of 5 specimens.

TABLE II

*Group II—10 Rabbits. Consecutive Values for Erythrocytes and Hemoglobin*

| Date | Red blood cells (000 omitted) | | | Hemoglobin | | |
|---|---|---|---|---|---|---|
| | Mean values | Standard deviation | Coefficient of variation | Mean values | Standard deviation | Coefficient of variation |
| *1928* | *per cmm.* | *per cmm.* | *per cent* | *per cent* | *per cent* | *per cent* |
| Mar. 29 | 4912 ± 87 | 407 | 8.29 | 66 ± 1.3 | 6 | 9.09 |
| Apr. 3 | 4899 ± 69 | 323 | 6.59 | 65 ± 1.4 | 4 | 6.15 |
| Apr 10 | 4842 ± 62 | 289 | 8.84 | 66 ± 1.0 | 5 | 4.55 |
| Apr. 17 | 5105 ± 163 | 762 | 14.93 | 64 ± 1.7 | 7 | 10.94 |
| Apr. 24 | 5197 ± 83 | 387 | 7.45 | 60 ± 1.7 | 7 | 11.67 |
| May 1 | 5169 ± 139 | 653 | 12.63 | 64 ± 1.7 | 7 | 10.94 |
| May 8 | 5348 ± 119 | 558 | 10.43 | 63 ± 1.3 | 6 | 9.52 |
| May 15 | 5047 ± 85 | 400 | 7.93 | 63 ± 1.7 | 6 | 9.52 |
| May 22 | 5242 ± 96 | 450 | 8.58 | 66 ± 1.7 | 7 | 10.61 |
| May 29 | 5270 ± 107 | 500 | 9.49 | 63 ± 1.7 | 7 | 11.11 |
| June 5 | 5648 ± 116 | 523 | 9.26 | 69 ± 1.3 | 6 | 8.70 |
| June 12 | 5329 ± 123 | 575 | 10.79 | 61 ± 1.3 | 6 | 9.84 |
| June 19 | 5151 ± 90 | 421 | 8.17 | 64 ± 1.7 | 8 | 12.50 |
| Mean | 5166 ± 39 | 209 | 4.05 | 64 ± 0.4 | 2 | 3.13 |
| Minimum | 4899 | | | 60 | | |
| Maximum | 5648 | | | 69 | | |

TABLE III

*Group III—10 Rabbits. Consecutive Values for Erythrocytes and Hemoglobin*

| Date | Red blood cells (000 omitted) | | | Hemoglobin | | |
|---|---|---|---|---|---|---|
| | Mean values | Standard deviation | Coefficient of variation | Mean values | Standard deviation | Coefficient of variation |
| *1928* | *per cmm.* | *per cmm.* | *per cent* | *per cent* | *per cent* | *per cent* |
| Sept. 20 | 5039 ± 34 | 161 | 3.20 | 70 ± 1.7 | 8 | 11.43 |
| Sept. 28 | 5129 ± 51 | 239 | 4.66 | 56 ± 1.0 | 5 | 8.93 |
| Oct. 10 | 5075 ± 23 | 106 | 2.09 | 70 ± 1.0 | 5 | 7.14 |
| Oct. 19 | 5116 ± 23 | 107 | 2.09 | 71 ± 1.7 | 8 | 11.27 |
| Nov. 2 | 5108 ± 51 | 240 | 4.70 | 56 ± 1.0 | 5 | 8.93 |
| Nov. 9 | 5108 ± 32 | 151 | 2.96 | 66 ± 1.4 | 7 | 10.61 |
| Nov. 16 | 5091 ± 41 | 191 | 3.75 | 65 ± 1.0 | 7 | 10.77 |
| Nov. 22 | 5252 ± 33 | 153 | 2.91 | 68 ± 1.3 | 6 | 8.82 |
| Mean | 5115 ± 15 | 62 | 1.21 | 65 ± 1.4 | 6 | 9.23 |
| Minimum | 5039 | | | 56 | | |
| Maximum | 5252 | | | 71 | | |

TABLE IV

*Group IV—10 Rabbits. Consecutive Values for Erythrocytes and Hemoglobin*

| Date | Red blood cells (000 omitted) | | | Hemoglobin | | |
|---|---|---|---|---|---|---|
| | Mean values | Standard deviation | Coefficient of variation | Mean values | Standard deviation | Coefficient of variation |
| *1928–29* | *per cmm.* | *per cmm.* | *per cent* | *per cent* | *per cent* | *per cent* |
| Nov. 27 | 5694 ± 141 | 660 | 11.59 | 68 ± 2.1 | 10 | 14.83 |
| Dec. 4 | 5274 ± 107 | 502 | 9.52 | 64 ± 1.4 | 7 | 10.28 |
| Dec. 11 | 5549 ± 127 | 595 | 10.72 | 67 ± 1.9 | 9 | 13.33 |
| Dec. 18 | 5375 ± 53 | 247 | 4.60 | 61 ± 1.2 | 6 | 9.50 |
| Dec. 26 | 5285 ± 83 | 389 | 7.35 | 69 ± 1.7 | 8 | 11.31 |
| Jan. 2 | 5274 ± 107 | 504 | 9.55 | 64 ± 2.0 | 9 | 14.68 |
| Jan. 8 | 4839 ± 65 | 306 | 6.32 | 55 ± 1.2 | 6 | 10.18 |
| Jan. 15 | 4860 ± 62 | 291 | 5.98 | 59 ± 1.3 | 6 | 10.61 |
| Jan. 22 | 4810 ± 44 | 206 | 4.27 | 59 ± 1.9 | 9 | 15.11 |
| Jan. 29 | 4941 ± 68 | 317 | 6.42 | 54 ± 1.0 | 5 | 8.25 |
| Feb. 5 | 4705 ± 69 | 322 | 6.84 | 58 ± 1.2 | 6 | 9.81 |
| Feb. 13 | 5028 ± 57 | 266 | 5.29 | 60 ± 1.1 | 5 | 8.60 |
| Feb. 19 | 4914 ± 86 | 405 | 8.24 | 61 ± 1.3 | 6 | 10.18 |
| Feb. 26 | 5034 ± 53 | 247 | 4.90 | 60 ± 1.2 | 6 | 9.16 |
| Mar. 12 | 4897 ± 52 | 244 | 4.98 | 63 ± 1.6 | 8 | 11.85 |
| Mar. 19 | 4943 ± 66 | 311 | 6.28 | 60 ± 0.9 | 4 | 7.05 |
| Mar. 26 | 4875 ± 61 | 284 | 5.83 | 59 ± 0.8 | 4 | 6.75 |
| Apr. 2 | 4820 ± 35 | 165 | 3.42 | 60 ± 1.2 | 5 | 9.00 |
| Apr. 9 | 4774 ± 55 | 256 | 5.35 | 58 ± 1.1 | 5 | 8.68 |
| Apr. 16 | 4849 ± 62 | 292 | 6.02 | 62 ± 1.4 | 7 | 10.50 |
| Apr. 23 | 4874 ± 49 | 229 | 4.69 | 61 ± 1.1 | 5 | 8.75 |
| Apr. 30 | 4806 ± 33 | 155 | 3.22 | 64 ± 1.1 | 5 | 7.94 |
| May 7 | 4907 ± 40 | 189 | 3.86 | 64 ± 0.7 | 3 | 5.06 |
| May 14 | 4816 ± 28 | 123 | 2.55 | 62 ± 1.6 | 7 | 11.34 |
| May 21 | 4808 ± 40 | 177 | 3.68 | 64 ± 1.4 | 6 | 9.91 |
| May 28 | 4843 ± 65 | 287 | 5.92 | 59 ± 1.7 | 8 | 13.05 |
| June 4 | 4843 ± 56 | 249 | 5.14 | 66 ± 1.8 | 8 | 11.82 |
| June 11 | 4936 ± 38 | 171 | 3.46 | 63 ± 2.4 | 11 | 17.00 |
| June 18 | 4888 ± 47 | 197 | 4.03 | 56 ± 1.4 | 6 | 10.16 |
| Mean | 4981 ± 31 | 245 | 4.93 | 61 ± 0.5 | 4 | 5.75 |
| Minimum | 4705 | | | 54 | | |
| Maximum | 5694 | | | 69 | | |

observed 8 to 35 weeks are presented in Tables I to V in the form of group means, together with the probable errors of the means, the standard deviations, and the coefficients of variation. The series of

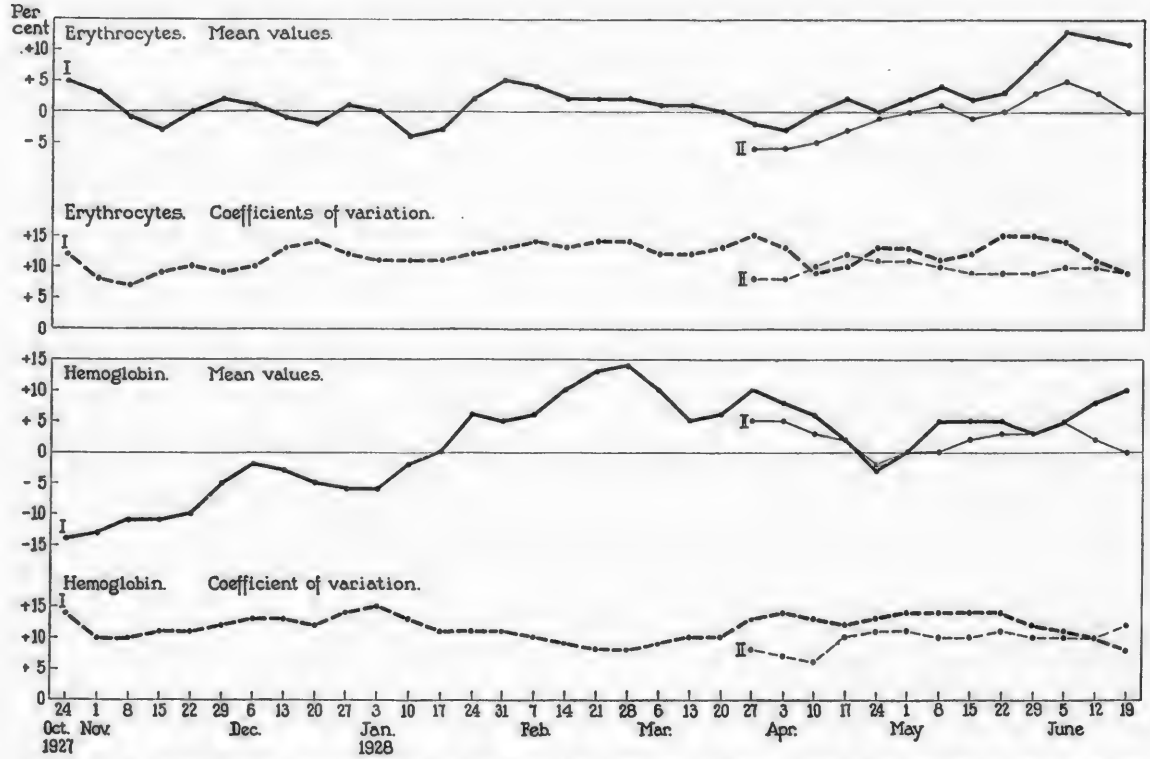
TABLE V

*Group V—5 Rabbits. Consecutive Values for Erythrocytes and Hemoglobin*

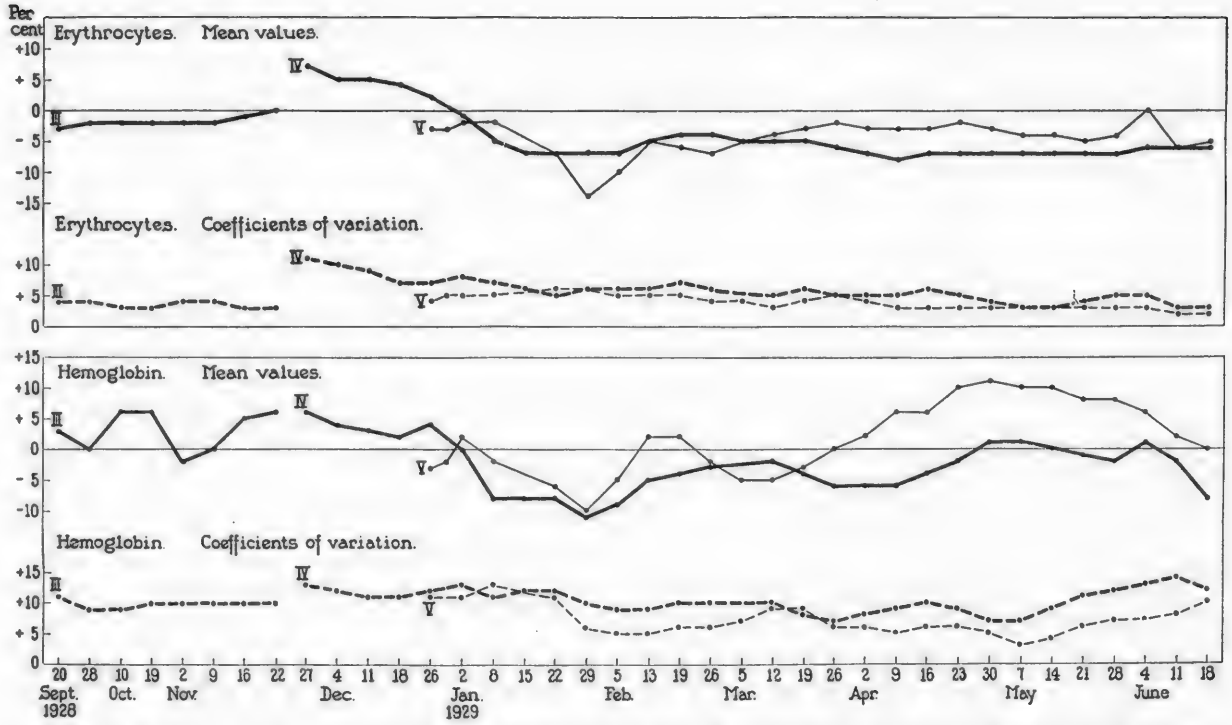
| Date | Red blood cells (000 omitted) | | | Hemoglobin | | |
|---|---|---|---|---|---|---|
| | Mean values | Standard deviation | Coefficient of variation | Mean values | Standard deviation | Coefficient of variation |
| *1928–29* | *per cmm.* | *per cmm.* | *per cent* | *per cent* | *per cent* | *per cent* |
| Dec. 29 | 5100 ± 57 | 190 | 2.73 | 60 ± 1.8 | 6 | 10.00 |
| Dec. 31 | 4920 ± 90 | 297 | 6.04 | 62 ± 2.4 | 8 | 12.90 |
| Jan. 3 | 5224 ± 84 | 279 | 5.34 | 64 ± 1.5 | 5 | 7.81 |
| Jan. 10 | 5108 ± 65 | 214 | 4.19 | 64 ± 3.3 | 11 | 17.19 |
| Jan. 24 | 4904 ± 82 | 271 | 5.53 | 57 ± 1.8 | 6 | 10.53 |
| Jan. 31 | 4384 ± 97 | 323 | 7.37 | 56 ± 0.6 | 2 | 3.57 |
| Feb 7 | 4592 ± 75 | 249 | 5.42 | 58 ± 0.9 | 3 | 5.17 |
| Feb. 15 | 5216 ± 54 | 178 | 3.41 | 67 ± 0.9 | 3 | 4.48 |
| Feb. 21 | 4730 ± 106 | 352 | 7.44 | 64 ± 1.2 | 4 | 6.25 |
| Mar. 1 | 4850 ± 35 | 117 | 2.41 | 61 ± 0.6 | 3 | 4.92 |
| Mar. 8 | 4894 ± 78 | 258 | 5.27 | 61 ± 1.2 | 4 | 6.56 |
| Mar. 15 | 5072 ± 37 | 124 | 2.44 | 58 ± 1.8 | 6 | 10.34 |
| Mar. 22 | 4990 ± 47 | 156 | 3.13 | 61 ± 1.8 | 6 | 9.84 |
| Mar. 29 | 5228 ± 86 | 286 | 5.47 | 64 ± 0.9 | 3 | 4.69 |
| Apr. 5 | 4940 ± 58 | 192 | 3.89 | 62 ± 1.2 | 4 | 6.45 |
| Apr. 12 | 5078 ± 50 | 164 | 3.23 | 69 ± 0.9 | 3 | 4.35 |
| Apr. 19 | 5030 ± 33 | 108 | 2.15 | 66 ± 1.2 | 4 | 6.06 |
| Apr. 26 | 5078 ± 57 | 189 | 3.72 | 70 ± 1.5 | 5 | 7.14 |
| May 3 | 5128 ± 32 | 105 | 2.05 | 71 ± 0.9 | 3 | 4.23 |
| May 10 | 4882 ± 60 | 198 | 4.06 | 68 ± 0.6 | 2 | 2.94 |
| May 17 | 4984 ± 39 | 130 | 2.61 | 70 ± 0.6 | 2 | 2.86 |
| May 24 | 4966 ± 49 | 161 | 3.24 | 67 ± 1.5 | 5 | 7.46 |
| May 31 | 4952 ± 42 | 138 | 2.79 | 67 ± 1.5 | 5 | 7.46 |
| June 7 | 5050 ± 42 | 140 | 2.77 | 70 ± 1.5 | 5 | 7.14 |
| June 14 | 4756 ± 29 | 96 | 2.02 | 60 ± 1.2 | 4 | 6.67 |
| June 21 | 5010 ± 33 | 108 | 2.16 | 64 ± 2.1 | 7 | 10.94 |
| Mean | 4964 ± 25 | 188 | 3.79 | 64 ± 0.5 | 4 | 6.25 |
| Minimum | 4384 | | | 56 | | |
| Maximum | 5228 | | | 71 | | |

curves in Text-figs. 1 and 2 are drawn as percentage deviations of the smoothed means from the standard values given above; other curves represent the smoothed values of the coefficients of variation of the means.

By using mean values to describe the results obtained, the trends or




TEXT-FIG. 1. Mean values for consecutive red blood cell and hemoglobin determinations as percentage deviations from standard values. 1927–28.




TEXT-FIG. 2. Mean values for consecutive red blood cell and hemoglobin determinations as percentage deviations from standard values. 1928–29.

changes characteristic of the group as a whole are shown. It is this feature of the results rather than the variations of individual animals which at present we wish to emphasize.

## DISCUSSION AND SUMMARY

In discussing the results of repeated observations on the erythrocytes and hemoglobin content of the peripheral blood of normal rabbits over long periods of time, it will be convenient to consider in chronological order the findings obtained in each of the five groups.

Group I, comprising 10 rabbits, was examined weekly from October 24, 1927 to June 21, 1928 (Table I). The mean numbers of red cells in 35 examinations were found to be comparatively constant.

The fluctuations in the curve of the percentage deviations of the smoothed means from a standard value of 5,200,000 cells per cubic millimeter (Text-fig. 1) do not vary more than 5 per cent above or below this figure until May and June when the curve rises to the 10 to 13 per cent level. In general, the curve describes 3 major swings, each occupying a period of approximately 3 months. From the October level of 5 per cent above the base line, there is an irregular fall to 4 per cent below it on January 10. During the succeeding 3 weeks, the curve rises abruptly to its initial level and very gradually descends to slightly below the base line (March 27 and April 3). During the last 3 months the curve rises to the highest levels observed although at its termination, its trend is again downward. The coefficients of variation for the red cells means (Table I) are fairly uniform, the curve of the smoothed values varying about the 10 per cent level (Text-fig. 1). The most irregular portion of the curve is its last quarter and this phase accompanied an augmentation of mean values. It will be noted that the changes in the trend of the red cell means at the end of January and the first of April are, in both instances, preceded by similar changes in the coefficient curve, at a 2 or 3 weeks' interval.

The range of mean hemoglobin values of Group I (Table I) was considerably greater than that of the erythrocyte means. The curve of these values in terms of the percentage variations of the smoothed means from a standard of 63 per cent fluctuates within a total range of 28 per cent, that is, 14 per cent above and below the base line (Text-fig. 1).

This curve can be divided into 3 major swings which, however, are not parallel with those of the red cells. From an initial low level the curve rises to 14 per cent above the base line by the end of February; the trend of the red cell curve in the

opposite direction ended the middle of January and for the next 3 observations, the direction of both curves is upward. From the end of February to the last of April, the hemoglobin curve is generally falling, while in May and June its general movement is again in an upward direction. In March, both the hemoglobin and red cell curves are falling and from the middle of April to the end of June both are rising. The coefficients of variation of the hemoglobin means (Table I) are comparable as far as order of magnitude is concerned, to those of the red cell means. From the curve representing the smoothed coefficients (Text-fig. 1) it will be noted that small values prevailed during January and February in which months the means were considerably increased. In like manner, the second period of higher hemoglobin mean values in May and June was characterized by a steady decline of the coefficient curve.

The second group of 10 rabbits (Group II) was examined during the last 3 months of the observation period of Group I (Table II). The values for the mean numbers of erythrocytes and for the mean hemoglobin contents occupy slightly lower levels than those for Group I (Text-fig. 1), but it will be noted that the curves representing these values are remarkably like those of Group I in general outline, the only important exception being in the case of the hemoglobin means during the last 2 weeks' observations.

It should be noted that in the corresponding portion of the curve representing the coefficients of variation of these means, an abrupt upward turn occurs indicating that the sharp fall in the mean values is due to certain individual findings and is not characteristic of the group as a whole. The coefficients of variations of both erythrocyte and hemoglobin means of Group II (Table II) are generally somewhat smaller than those of Group I as is illustrated by the curves shown in Text-fig. 1.

The third group of 10 rabbits (Group III) was examined in September, October, and November of 1928. The erythrocytes and the hemoglobin means, together with their coefficients of variations are given in Table III and the curves representing these values appear in Text-fig. 2.

In the case of the red blood cells, the means were extremely regular at a level slightly below the standard value while the curve of their smoothed coefficients of variation is steadily maintained at the 3 to 4 per cent level. The hemoglobin means, on the other hand, were less uniform and their curve describes several fluctuations which, however, are of a much smaller magnitude than those of Group I. In contrast to these variations, however, the curve of the smoothed coefficients of variation of these means is almost a straight line at the 10 per cent level, a value slightly smaller than that which generally obtained in Group I.

Examination of the fourth group of 10 rabbits was begun in November, 1928 and of the fifth group comprising 5 rabbits in December, 1928; both groups were observed to the end of June, 1929. The erythrocyte and hemoglobin means and their coefficients of variations are given in Tables IV and V, and the curves illustrating these values in Text-fig. 2.

In the case of Group IV, the red cell curve which shows practically no irregularities, describes during the first 2 months' observation a steady downward trend from an initial 7 per cent above to 7 per cent below the standard value; during the remainder of the experiment this low level is almost perfectly maintained. The curve of the smoothed coefficients of variation of the red cell means has a similar form; from an initial 11 per cent level, it falls to 5 per cent at the end of the second month and to 3 per cent in June.

The hemoglobin means of Group IV were much less uniform than the red cell values as was the case with the previous groups; the fluctuations of the curve representing these values (Text-fig. 2) vary between 6 per cent above to 11 per cent below the base line. During the first 2 months, the direction of the curve is downward, parallel to that of the red cells, but from this time onward, while the red cell means continue at a fairly constant level, the general trend of the hemoglobin means is upward. The rise is interrupted in March and again in June; on the first occasion there is also a fall in the number of red cells but on the second, the red cell level is unchanged. The smoothed coefficients of variation of the hemoglobin means for Group IV vary from 7 to 14 per cent (Text-fig. 2). Their curve is fairly stationary at the 11 to 12 per cent level during the first 2 months' observations; in subsequent months somewhat lower and more irregular values obtain, and in May and June a rise to 12 and 14 per cent is observed.

The curve representing the erythrocyte means of Group V differs but little from that of Group IV as shown in Text-fig. 2. It begins in December at approximately the same level which Group IV occupies at this time and ends at the same level in June. There is an initial abrupt fall to 14 per cent below the base line (end of January) which is followed by a sharp rise, and from then onward, a fairly constant level is maintained at 5 per cent below the standard value. During January and February, the general level of the curve is slightly lower than that of Group IV while in March, April, May, and June, the relation is reversed. On the whole, the curve is uniform and contains but two irregular portions, one the end of January when the downward trend is prolonged to the lowest level observed in any of the 5 groups, that is, 13 per cent below the standard value, and the other the first of June at which time there is a sharp but temporary upward movement. The curve representing the smoothed coefficients of variation of the red cell means which is extremely uniform (Text-fig. 2) occupies a general level slightly below that of Group IV. It describes a gradual fall from 6 to 2 per cent.

In the case of the hemoglobin content, the mean values of Group V were somewhat higher than those of Group IV, but the direction of the variations observed

was essentially the same (Text-fig. 2). The curves representing these values are similar in general contour with parallel fluctuations but those of Group IV are somewhat more pronounced, their extremes being 10 per cent below and 11 per cent above the standard value. The level of the curve representing the smoothed coefficients of variation of the hemoglobin means of Group V is generally lower than that of Group IV, as was the case with the red cells (Text-fig. 2); the fluctuations in magnitude vary from 3 to 13 per cent. The general trend of this curve is similar to that of Group IV.

The great majority of the rabbits in these experiments were in excellent physical condition and gained in weight during the period of observation. There were two deaths, both in Group IV, which occurred toward the end of the experiment. Both animals had had marked snuffles and at autopsy, an extensive purulent exudate was found in the nasal passages and sinuses and in one rabbit, there was also a chronic diffuse interstitial nephritis. Snuffles and ear canker of various grades which were observed in certain rabbits will be referred to in a subsequent paper in connection with the discussion of the observations on the neutrophile cells (4).

There are certain features of these consecutive observations on the peripheral blood of normal rabbits which may now be briefly considered. In the first place, the weekly range of variation of the erythrocyte mean counts was considerably smaller than that of the hemoglobin means, a result which indicates that under the conditions of these experiments, the hemoglobin was the more labile of these two blood constituents. The technic employed may have contributed to this finding in that the error involved in a colorimetric method may be greater than in one of cell enumeration. With each specimen of blood, at least 3 hemoglobin readings were made and the mean value used; and in addition, duplicate readings with another instrument have always checked with those of our own. Secondly, it was found that a brief or a more sustained alteration in the erythrocyte means was not necessarily accompanied by a shift of the hemoglobin level in the opposite direction. In certain instances, such a change was observed, but in others, both values moved in the same direction and in still others, the hemoglobin means pursued a more or less protracted rise while the numbers of red cells were maintained at a fairly stationary level. Thirdly, the most irregular results with respect to both red cell and hemoglobin means were found in Group I and in addition,

their standard deviations were on the whole, of a higher order of magnitude than those of the other groups (Tables I to V). The examinations on Group I were not all made on the same day, half the animals being examined on one day and half on the following day; in the case of the other groups, all animals were examined on the same day. As has been mentioned in the section on Materials and Methods, the rabbits of Group I were also subjected to additional bleedings for purposes of chemical examination. Although this procedure was carried out most frequently in the first 2 months of the experiment, there is no striking difference in the order of magnitude of the standard deviations of the means nor in the coefficients of variations during this period as compared with later months (Table I). It is probable, therefore, that the greater irregularities of mean values and the higher standard deviations noted in Group I were related to the divided time of the blood counts themselves or to some other factor rather than to the additional bleedings for chemical examination.

In the present consideration, the major changes in mean values as observed over several weeks or months have been emphasized rather than fluctuations occurring in comparatively brief periods. Analysis of the results from the standpoint of the ratio of the difference of the mean values to their probable errors indicates that the major changes observed in both the red cell and hemoglobin levels are of statistical significance, as shown by the following examples:

| Group number | Red blood cells | | | Hemoglobin | | |
|---|---|---|---|---|---|---|
| | Dates of mean values | | Ratio | Dates of mean values | | Ratio |
| I | Jan. 10 | Jan. 24 | 3.65 | Oct. 24 | Jan. 10 | 2.94 |
| | Apr. 3 | June 19 | 5.44 | Apr. 24 | June 19 | 5.24 |
| II | Mar. 29 | May 8 | 2.96 | Mar. 29 | Apr. 24 | 2.73 |
| | Apr. 3 | June 12 | 3.05 | Apr. 24 | June 5 | 4.09 |
| III | Sept. 20 | Nov. 22 | 4.53 | Sept. 20 | Nov. 2 | 7.00 |
| IV | Nov. 27 | June 18 | 5.38 | Nov. 27 | June 18 | 4.80 |
| | Feb. 26 | Apr. 30 | 3.68 | Dec. 11 | Jan. 22 | 2.96 |
| V | Dec. 29 | Feb. 7 | 5.40 | Jan. 24 | Apr. 19 | 4.09 |
| | Feb. 21 | Apr. 26 | 3.25 | Apr. 26 | June 14 | 5.26 |

A striking feature of these results is the degree of parallelism shown by two groups of animals examined over the same period with respect to the fluctuations of erythrocytes and hemoglobin mean values. The curves in Text-figs. 1 and 2 show that the trend of values for one group of animals is almost always reflected in a similar trend for the other group.

Finally, these observations make it clearly evident that the results obtained on the groups of rabbits examined in one year may not be entirely similar to those obtained on other groups followed for similar periods in another year. In the case of both the erythrocytes and the hemoglobin, the mean values during 1927–28 were generally higher than those of 1928–29. Furthermore, there was a definite trend on the part of both red cell and hemoglobin means toward higher values in the spring and early summer months of the first year which were not observed in the following year. In the fall and early winter months of both years, on the other hand, the red cells tended toward smaller values; this was also the case for the hemoglobin in the second but not in the first year.

## CONCLUSIONS

Observations are reported on the consecutive weekly erythrocyte counts and the hemoglobin contents of the peripheral blood in 5 groups of normal rabbits, comprising 45 animals, during a period of 20 months from October, 1927 to July, 1929. The duration of individual group examinations varied from 8 to 35 weeks. The results are analyzed on the basis of the weekly mean values of each group.

On the whole, the erythrocyte values were quite uniform within a narrow range of variation, while the hemoglobin content was comparatively irregular within a wider range of variation. The major changes in the levels of mean values of both the red cells and the hemoglobin, however, were found to be statistically significant.

The directions or trends in the levels of the erythrocyte and hemoglobin mean values did not necessarily move in opposite directions.

The general levels of the erythrocyte and hemoglobin mean values were not identical for two consecutive years, those of 1927–28 being higher than those of 1928–29.

The fluctuations of both red cell and hemoglobin mean values

observed in one group of animals were also usually observed in another group examined during the same months.

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# STUDIES IN THE BLOOD CYTOLOGY OF THE RABBIT

## III. Consecutive Total White Blood Cell Observations on Groups of Normal Rabbits

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In the preceding paper of this series, experiments dealing with consecutive observations on the peripheral cytology of normal rabbits for prolonged periods of time were described and the results pertaining to the erythrocytes and the hemoglobin were reported (1). The observations were made on 5 groups of male rabbits, 4 of 10 and 1 of 5 animals, during a period extending from October, 1927 to July, 1929. In the present paper, the results relating to the total white cell counts are reported together with a consideration of these values from the standpoint of the total granular leucocytes—the neutrophiles (pseudo-eosinophiles), the basophiles, and the eosinophiles—on the one hand, and the total non-granular cells—the lymphocytes and the monocytes—on the other.

### *Materials and Methods*

Since the conduct of the experiments and the methods employed in analyzing the results obtained have previously been described (1), it will suffice here merely to state that in the great majority of cases the examination of the blood was carried out at weekly intervals. The differential counts which were made by means of the supravital neutral red technic, were based upon counts of 100 cells for each specimen.

The number of examinations on each group of rabbits was: Group I, 35; Group II, 13; Group III, 8; Group IV, 29; Group V, 26. The first 4 groups comprised 10, and the last, 5 animals.

### RESULTS

The results on consecutive total white blood cell counts in 5 groups of normal rabbits observed 8 to 35 weeks are given in Tables I to V and

TABLE I

*Group I—10 Rabbits. Consecutive Values for Total White Blood Cells and for Total Granular and Non-Granular Cells*

| Date | Total white blood cells | | | Granular: Neutrophiles, basophiles, and eosinophiles | | | Non-granular: Lymphocytes and monocytes | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean values | Standard deviation | Coefficient of variation | Mean values | Standard deviation | Coefficient of variation | Mean values | Standard deviation | Coefficient of variation |
| *1927–28* | *per cmm.* | *per cmm.* | *per cent* | *per cmm.* | *per cmm.* | *per cent* | *per cmm.* | *per cmm.* | *per cent* |
| Oct. 24* | 8825 ± 401 | 1879 | 21.29 | 5586 ± 261 | 1224 | 21.91 | 3240 ± 243 | 1138 | 35.12 |
| Nov. 1 | 8245 ± 430 | 2015 | 24.44 | 5293 ± 304 | 1423 | 26.89 | 2953 ± 188 | 879 | 29.77 |
| Nov. 8** | 8030 ± 507 | 2376 | 29.59 | 4948 ± 283 | 1328 | 26.84 | 3082 ± 261 | 1222 | 39.65 |
| Nov. 15 | 8555 ± 275 | 1288 | 15.06 | 4910 ± 192 | 901 | 18.35 | 3645 ± 132 | 618 | 16.95 |
| Nov. 22 | 9935 ± 601 | 2816 | 28.34 | 6522 ± 355 | 1666 | 25.54 | 3414 ± 224 | 1048 | 30.70 |
| Nov. 29 | 9685 ± 747 | 3504 | 36.18 | 5494 ± 578 | 2710 | 49.33 | 4192 ± 265 | 1241 | 29.60 |
| Dec. 6 | 8840 ± 463 | 2169 | 24.54 | 4987 ± 395 | 1852 | 37.14 | 3854 ± 177 | 831 | 21.56 |
| Dec. 13 | 9130 ± 756 | 3546 | 38.84 | 4783 ± 393 | 1841 | 38.49 | 4348 ± 469 | 2198 | 50.55 |
| Dec. 20 | 9690 ± 582 | 2729 | 28.16 | 6251 ± 417 | 1955 | 31.27 | 3439 ± 196 | 920 | 26.75 |
| Dec. 27 | 9400 ± 380 | 1780 | 18.94 | 5983 ± 258 | 1209 | 20.21 | 3418 ± 200 | 936 | 27.38 |
| Jan. 3 | 9800 ± 469 | 2200 | 22.45 | 5811 ± 254 | 1192 | 20.51 | 3990 ± 347 | 1628 | 40.80 |
| Jan. 10 | 10240 ± 440 | 2065 | 20.17 | 5827 ± 338 | 1586 | 27.22 | 4392 ± 255 | 1195 | 27.21 |
| Jan. 17 | 10375 ± 613 | 2873 | 27.69 | 5899 ± 496 | 2327 | 39.45 | 4454 ± 345 | 1616 | 36.28 |
| Jan. 24 | 10380 ± 481 | 2253 | 21.70 | 5891 ± 277 | 1301 | 22.08 | 4478 ± 260 | 1217 | 27.18 |
| Jan. 31 | 11081 ± 718 | 3367 | 30.39 | 6590 ± 520 | 2439 | 37.01 | 4466 ± 335 | 1571 | 35.18 |
| Feb. 7 | 11095 ± 651 | 3053 | 27.52 | 6477 ± 544 | 2550 | 39.37 | 4601 ± 240 | 1123 | 24.41 |
| Feb. 14 | 10920 ± 671 | 3146 | 28.81 | 6643 ± 554 | 2595 | 39.06 | 4270 ± 77 | 362 | 8.48 |
| Feb. 21 | 12450 ± 504 | 2365 | 19.00 | 6545 ± 353 | 1654 | 25.27 | 5888 ± 246 | 1152 | 19.57 |
| Feb. 28 | 11700 ± 913 | 4281 | 36.59 | 8052 ± 842 | 3950 | 49.06 | 3651 ± 65 | 303 | 8.30 |
| Mar. 6 | 10455 ± 494 | 2316 | 22.15 | 6390 ± 329 | 1543 | 24.15 | 4051 ± 230 | 1078 | 26.61 |
| Mar. 13 | 9910 ± 657 | 3079 | 31.07 | 6186 ± 505 | 2368 | 38.28 | 3715 ± 276 | 1296 | 34.89 |
| Mar. 20 | 11270 ± 792 | 3711 | 32.93 | 7164 ± 760 | 3561 | 46.77 | 3647 ± 170 | 795 | 21.80 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Mar. 27 | 10230 ± 499 | 2338 | 22.85 | 6062 ± 372 | 1742 | 28.74 | 4156 ± 235 | 1100 | 26.47 |
| Apr. 3 | 10750 ± 620 | 2908 | 27.16 | 6202 ± 429 | 2013 | 32.46 | 4503 ± 397 | 1859 | 41.28 |
| Apr. 10 | 12275 ± 679 | 3183 | 25.93 | 7271 ± 464 | 2174 | 29.90 | 4992 ± 221 | 1038 | 20.79 |
| Apr. 17 | 11225 ± 543 | 2547 | 22.69 | 6385 ± 379 | 1779 | 27.86 | 4840 ± 255 | 1195 | 24.69 |
| Apr. 24 | 10535 ± 718 | 3367 | 31.96 | 6522 ± 561 | 2629 | 40.31 | 4006 ± 315 | 1475 | 36.84 |
| May 1 | 11240 ± 570 | 2670 | 23.75 | 6130 ± 303 | 1422 | 23.20 | 5098 ± 301 | 1411 | 27.68 |
| May 8 | 11100 ± 726 | 3404 | 30.67 | 6644 ± 481 | 2255 | 33.94 | 4439 ± 309 | 1448 | 32.62 |
| May 15 | 11605 ± 504 | 2364 | 20.37 | 6761 ± 388 | 1819 | 26.90 | 4845 ± 199 | 933 | 19.26 |
| May 22 | 12085 ± 698 | 3273 | 27.08 | 7217 ± 570 | 2670 | 37.00 | 4869 ± 232 | 1088 | 22.35 |
| May 29 | 12035 ± 901 | 4226 | 35.11 | 6443 ± 369 | 1732 | 26.88 | 5447 ± 594 | 2787 | 51.17 |
| June 5 | 13590 ± 532 | 2495 | 18.36 | 7445 ± 312 | 1461 | 19.62 | 6144 ± 321 | 1504 | 24.48 |
| June 12 | 12380 ± 797 | 3735 | 30.17 | 6656 ± 330 | 1547 | 23.24 | 5725 ± 474 | 2221 | 38.79 |
| June 19 | 11455 ± 831 | 3895 | 34.00 | 6973 ± 497 | 2332 | 33.44 | 4482 ± 402 | 1883 | 40.90 |
| Mean | 10585 ± 146 | 1281 | 12.10 | 6269 ± 87 | 759 | 12.11 | 4307 ± 87 | 763 | 17.72 |
| Minimum | 8030 | | | 4783 | | | 2953 | | |
| Maximum | 13590 | | | 8052 | | | 6144 | | |

* October 24 and 26.

** November 4 and 9.

TABLE II

*Group II—10 Rabbits. Consecutive Values for Total White Blood Cells and for Total Granular and Non-Granular Cells*

| Date | Total white blood cells | | | Granular: Neutrophiles, basophiles, and eosinophiles | | | Non-granular: Lymphocytes and monocytes | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean values | Standard deviation | Coefficient of variation | Mean values | Standard deviation | Coefficient of variation | Mean values | Standard deviation | Coefficient of variation |
| *1928* | *per cmm.* | *per cmm.* | *per cent* | *per cmm.* | *per cmm.* | *per cent* | *per cmm.* | *per cmm.* | *per cent* |
| Mar. 29 | 7095 ± 275 | 1290 | 18.18 | 4300 ± 257 | 1206 | 28.05 | 2783 ± 185 | 866 | 31.12 |
| Apr. 3 | 8735 ± 422 | 1979 | 22.66 | 4745 ± 205 | 962 | 20.07 | 3990 ± 287 | 1345 | 33.71 |
| Apr. 10 | 8445 ± 534 | 2504 | 29.65 | 4516 ± 346 | 1622 | 35.92 | 3917 ± 320 | 1498 | 38.24 |
| Apr. 17 | 10150 ± 531 | 2487 | 24.50 | 5640 ± 352 | 1652 | 29.29 | 2405 ± 348 | 1631 | 36.20 |
| Apr. 24 | 9350 ± 525 | 2462 | 26.33 | 5430 ± 367 | 1720 | 31.68 | 3900 ± 240 | 1126 | 28.72 |
| May 1 | 8040 ± 315 | 1477 | 18.37 | 4103 ± 209 | 978 | 23.84 | 3927 ± 237 | 1109 | 28.17 |
| May 8 | 9085 ± 394 | 1845 | 20.31 | 5093 ± 299 | 1401 | 27.51 | 3992 ± 233 | 1092 | 27.35 |
| May 15 | 7610 ± 579 | 2716 | 35.69 | 4285 ± 390 | 1829 | 42.68 | 3325 ± 276 | 1293 | 38.89 |
| May 22 | 11120 ± 449 | 2107 | 18.95 | 6132 ± 351 | 1645 | 26.83 | 4988 ± 279 | 1308 | 26.22 |
| May 29 | 9460 ± 702 | 3289 | 34.77 | 4847 ± 313 | 1465 | 30.22 | 4601 ± 416 | 1949 | 42.36 |
| June 5 | 10230 ± 445 | 2088 | 20.41 | 5570 ± 317 | 1485 | 26.66 | 4660 ± 283 | 1325 | 28.43 |
| June 12 | 9030 ± 557 | 2611 | 28.91 | 4772 ± 254 | 1190 | 25.20 | 4229 ± 323 | 1514 | 35.22 |
| June 19 | 10355 ± 375 | 1756 | 16.96 | 5663 ± 267 | 1250 | 22.07 | 4692 ± 234 | 1097 | 23.38 |
| Mean | 9131 ± 208 | 1114 | 12.20 | 5007 ± 114 | 609 | 12.16 | 3958 ± 135 | 722 | 18.24 |
| Minimum | 7095 | | | 4103 | | | 2405 | | |
| Maximum | 11120 | | | 6132 | | | 4988 | | |

TABLE III

*Group III—10 Rabbits. Consecutive Values for Total White Blood Cells and for Total Granular and Non-Granular Cells*

| Date | Total white blood cells | | | Granular: Neutrophiles, basophiles, and eosinophiles | | | Non-granular: Lymphocytes and monocytes | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean values | Standard deviation | Coefficient of variation | Mean values | Standard deviation | Coefficient of variation | Mean values | Standard deviation | Coefficient of variation |
| *1928* | *per cmm.* | *per cmm.* | *per cent* | *per cmm.* | *per cmm.* | *per cent* | *per cmm.* | *per cmm.* | *per cent* |
| Sept. 20 | 8040 ± 672 | 3152 | 39.20 | 5337 ± 355 | 1662 | 31.02 | 2683 ± 281 | 1319 | 49.16 |
| Sept. 28 | 7030 ± 474 | 2223 | 31.62 | 3796 ± 176 | 826 | 21.76 | 3234 ± 292 | 1368 | 42.30 |
| Oct. 10 | 7715 ± 487 | 2282 | 29.58 | 4779 ± 427 | 2000 | 41.68 | 2916 ± 99 | 464 | 15.91 |
| Oct. 19 | 6985 ± 381 | 1785 | 25.55 | 3675 ± 118 | 553 | 15.05 | 3310 ± 207 | 969 | 29.27 |
| Nov. 2 | 7560 ± 337 | 1581 | 20.91 | 4754 ± 204 | 955 | 20.09 | 2806 ± 101 | 473 | 16.86 |
| Nov. 9 | 7885 ± 297 | 1393 | 17.67 | 4713 ± 292 | 1370 | 29.07 | 3173 ± 113 | 783 | 24.68 |
| Nov. 16 | 6595 ± 270 | 1264 | 19.17 | 3940 ± 144 | 674 | 17.11 | 2655 ± 176 | 824 | 31.04 |
| Nov. 22 | 7165 ± 314 | 1473 | 20.56 | 3922 ± 168 | 788 | 20.09 | 3243 ± 196 | 921 | 28.40 |
| Mean | 7372 ± 112 | 471 | 6.39 | 4365 ± 135 | 566 | 12.97 | 3003 ± 60 | 251 | 8.36 |
| Minimum | 6985 | | | 3675 | | | 2655 | | |
| Maximum | 8040 | | | 5337 | | | 3310 | | |

TABLE IV

*Group IV—10 Rabbits. Consecutive Values for Total White Blood Cells and for Total Granular and Non-Granular Cells*

| Date | Total white blood cells | | | Granular: Neutrophiles, basophiles, and eosinophiles | | | Non-granular: Lymphocytes and monocytes | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean values | Standard deviation | Coefficient of variation | Mean values | Standard deviation | Coefficient of variation | Mean values | Standard deviation | Coefficient of variation |
| *1928–29* | *per cmm.* | *per cmm.* | *per cent* | *per cmm.* | *per cmm.* | *per cent* | *per cmm.* | *per cmm.* | *per cent* |
| Nov. 27 | 7755 ± 201 | 1605 | 20.69 | 4593 ± 265 | 1243 | 27.07 | 3162 ± 195 | 918 | 29.03 |
| Dec. 4 | 7665 ± 357 | 1673 | 21.82 | 4453 ± 308 | 1445 | 32.44 | 3212 ± 162 | 762 | 23.72 |
| Dec. 11 | 7245 ± 438 | 2054 | 28.34 | 4467 ± 308 | 1446 | 32.37 | 2771 ± 216 | 1012 | 36.51 |
| Dec. 18 | 6660 ± 430 | 2014 | 30.23 | 3670 ± 198 | 926 | 25.23 | 2958 ± 291 | 1365 | 46.13 |
| Dec. 26 | 6720 ± 357 | 1673 | 24.88 | 4192 ± 281 | 1319 | 31.46 | 2522 ± 143 | 679 | 26.91 |
| Jan. 2 | 6340 ± 237 | 1111 | 17.52 | 4320 ± 209 | 979 | 22.66 | 2019 ± 81 | 390 | 19.32 |
| Jan. 8 | 6125 ± 403 | 1890 | 30.86 | 3595 ± 326 | 1527 | 42.48 | 2530 ± 166 | 790 | 31.21 |
| Jan. 15 | 6620 ± 270 | 1265 | 19.10 | 4087 ± 221 | 1035 | 25.31 | 2518 ± 96 | 458 | 18.20 |
| Jan. 22 | 6645 ± 431 | 2021 | 30.41 | 4102 ± 274 | 1283 | 31.28 | 2543 ± 201 | 953 | 37.47 |
| Jan. 29 | 8570 ± 462 | 2166 | 25.27 | 5510 ± 322 | 1509 | 27.37 | 3061 ± 179 | 851 | 27.80 |
| Feb. 5 | 6535 ± 412 | 1932 | 29.56 | 4116 ± 325 | 1526 | 37.07 | 2419 ± 186 | 884 | 36.53 |
| Feb. 13 | 8840 ± 550 | 2579 | 29.17 | 5978 ± 391 | 1833 | 30.66 | 2862 ± 207 | 980 | 34.24 |
| Feb. 19 | 9890 ± 352 | 1648 | 16.66 | 7050 ± 319 | 1495 | 21.19 | 2800 ± 159 | 757 | 27.04 |
| Feb. 26 | 9515 ± 418 | 1960 | 20.59 | 6364 ± 359 | 1683 | 26.44 | 3104 ± 141 | 673 | 21.67 |
| Mar. 12 | 7785 ± 261 | 1224 | 15.71 | 4731 ± 186 | 873 | 18.44 | 3013 ± 195 | 922 | 30.61 |
| Mar. 19 | 9305 ± 484 | 2267 | 24.36 | 6259 ± 423 | 1982 | 31.66 | 2046 ± 175 | 829 | 27.20 |
| Mar. 26 | 8875 ± 501 | 2349 | 26.46 | 5926 ± 371 | 1741 | 29.37 | 2925 ± 237 | 1119 | 38.26 |
| Apr. 2 | 8475 ± 520 | 2436 | 28.74 | 6058 ± 398 | 1865 | 30.78 | 2417 ± 218 | 1032 | 42.69 |
| Apr. 9 | 9365 ± 343 | 1610 | 17.19 | 5686 ± 146 | 685 | 12.04 | 3596 ± 240 | 1136 | 46.98 |
| Apr. 16 | 9360 ± 470 | 2204 | 23.54 | 5886 ± 326 | 1527 | 25.94 | 3474 ± 252 | 1291 | 37.16 |
| Apr. 23 | 10185 ± 440 | 2062 | 20.24 | 6246 ± 254 | 1193 | 19.10 | 3887 ± 249 | 1276 | 32.82 |
| Apr. 30 | 8840 ± 347 | 1625 | 18.38 | 5912 ± 244 | 1144 | 19.36 | 2920 ± 214 | 1113 | 38.09 |

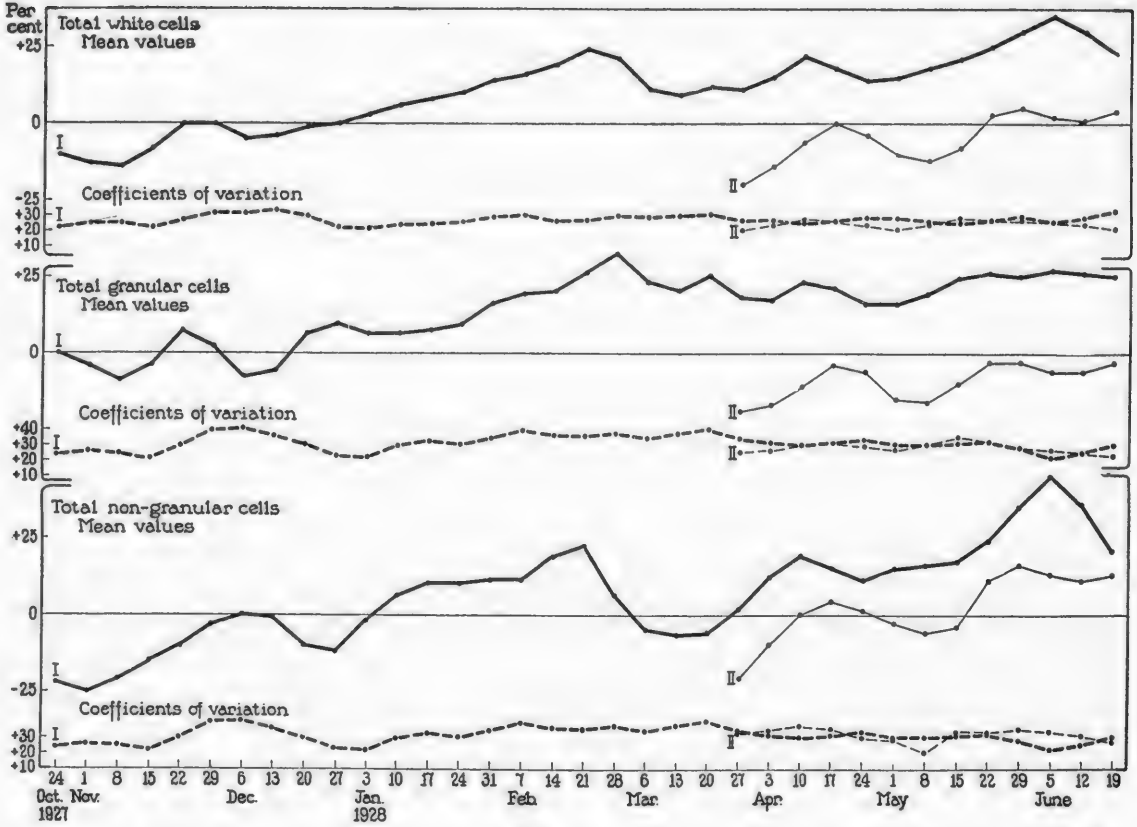
| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| May 7 | 9375 ± 259 | 1212 | 12.92 | 6066 ± 215 | 1010 | 16.64 | 3273 ± 133 | 732 | 22.37 |
| May 14 | 8067 ± 342 | 1523 | 18.87 | 4901 ± 232 | 1086 | 22.14 | 3136 ± 134 | 739 | 23.57 |
| May 21 | 7478 ± 275 | 1223 | 16.35 | 5241 ± 236 | 1106 | 21.11 | 2237 ± 81 | 490 | 21.89 |
| May 28 | 8439 ± 351 | 1560 | 18.48 | 5421 ± 273 | 1278 | 23.67 | 3002 ± 127 | 707 | 23.54 |
| June 4 | 7183 ± 313 | 1392 | 19.38 | 4545 ± 269 | 1260 | 27.71 | 2638 ± 134 | 739 | 28.00 |
| June 11 | 7006 ± 350 | 1557 | 22.22 | 4577 ± 237 | 1111 | 24.27 | 2429 ± 96 | 562 | 23.12 |
| June 18 | 7031 ± 265 | 1113 | 15.83 | 3883 ± 162 | 758 | 19.53 | 3139 ± 192 | 1011 | 32.20 |
| Mean | 7996 ± 148 | 1183 | 14.79 | 5098 ± 130 | 1039 | 20.38 | 2883 ± 53 | 411 | 14.30 |
| Minimum | 6125 | | | 3595 | | | 2019 | | |
| Maximum | 10185 | | | 7050 | | | 3887 | | |

TABLE V

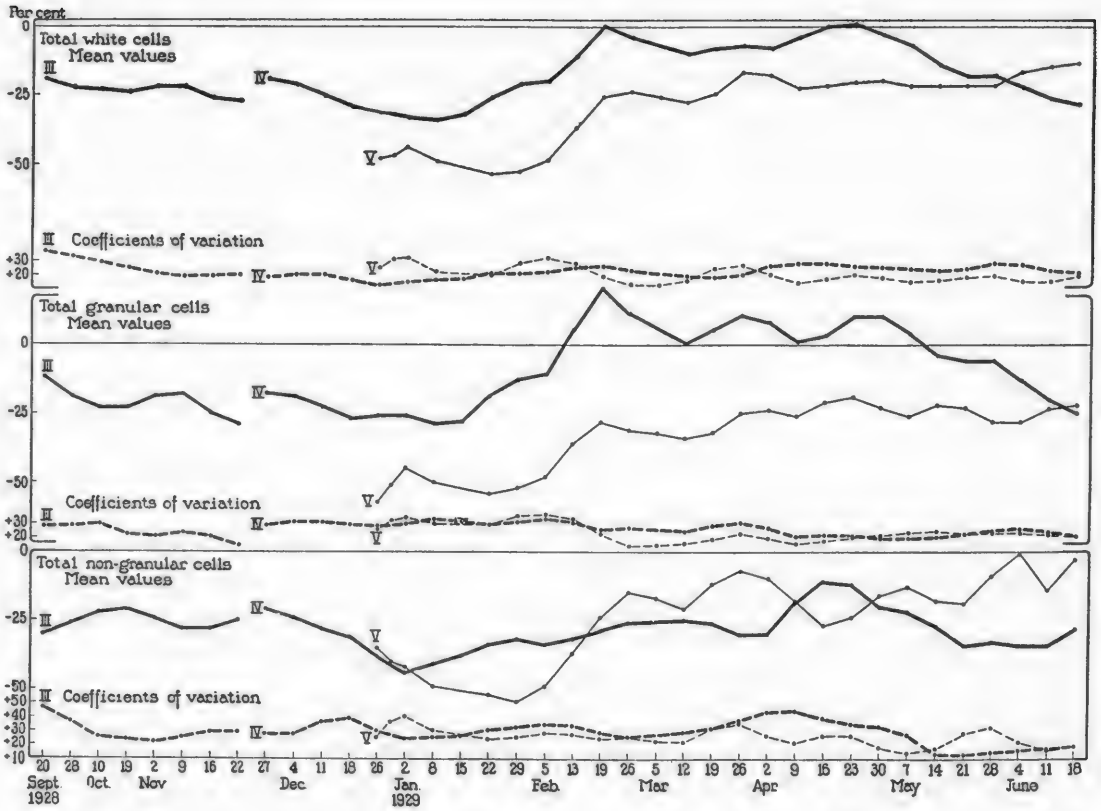
*Group V—5 Rabbits. Consecutive Values for Total White Blood Cells and for Total Granular and Non-Granular Cells*

| Date | Total white blood cells | | | Granular: Neutrophiles, basophiles, and eosinophiles | | | Non-granular: Lymphocytes and monocytes | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean values | Standard deviation | Coefficient of variation | Mean values | Standard deviation | Coefficient of variation | Mean values | Standard deviation | Coefficient of variation |
| *1928–29* | *per cmm.* | *per cmm.* | *per cent* | *per cmm.* | *per cmm.* | *per cent* | *per cmm.* | *per cmm.* | *per cent* |
| Dec. 29 | 5310 ± 338 | 1119 | 21.07 | 2369 ± 178 | 591 | 24.95 | 2941 ± 177 | 586 | 19.93 |
| Dec. 31 | 4410 ± 441 | 1463 | 33.17 | 2390 ± 246 | 817 | 34.18 | 2020 ± 222 | 736 | 36.44 |
| Jan. 3 | 6260 ± 714 | 2367 | 37.81 | 3555 ± 371 | 1231 | 34.63 | 2697 ± 421 | 1396 | 51.76 |
| Jan. 10 | 4510 ± 248 | 822 | 18.23 | 2514 ± 255 | 844 | 33.57 | 1996 ± 121 | 402 | 20.14 |
| Jan. 24 | 4110 ± 138 | 458 | 11.14 | 2431 ± 131 | 433 | 17.81 | 1679 ± 150 | 496 | 29.54 |
| Jan. 31 | 4820 ± 549 | 1820 | 37.76 | 2812 ± 404 | 1340 | 47.65 | 2008 ± 125 | 414 | 20.62 |
| Feb. 7 | 4240 ± 322 | 1066 | 25.14 | 2451 ± 186 | 617 | 25.17 | 1789 ± 161 | 532 | 29.74 |
| Feb. 15 | 6400 ± 757 | 2509 | 39.20 | 3648 ± 506 | 1677 | 45.97 | 2752 ± 271 | 898 | 32.63 |
| Feb. 21 | 7260 ± 219 | 725 | 9.99 | 4294 ± 200 | 662 | 15.42 | 2966 ± 122 | 405 | 13.65 |
| Mar. 1 | 7210 ± 347 | 1149 | 15.94 | 3547 ± 115 | 380 | 10.71 | 3663 ± 390 | 1294 | 35.33 |
| Mar. 8 | 7380 ± 203 | 672 | 9.11 | 3921 ± 168 | 556 | 14.18 | 3459 ± 179 | 593 | 17.14 |
| Mar. 15 | 6510 ± 335 | 1111 | 17.07 | 3562 ± 163 | 540 | 15.16 | 2948 ± 171 | 567 | 19.23 |
| Mar. 22 | 7010 ± 459 | 1521 | 21.70 | 3594 ± 166 | 550 | 15.30 | 3426 ± 311 | 1031 | 30.09 |
| Mar. 29 | 8320 ± 855 | 2833 | 34.05 | 4323 ± 361 | 1195 | 27.64 | 3997 ± 522 | 1730 | 43.28 |
| Apr. 5 | 8070 ± 430 | 1425 | 17.66 | 4360 ± 238 | 789 | 18.10 | 3710 ± 262 | 867 | 23.37 |
| Apr. 12 | 6960 ± 209 | 692 | 9.94 | 3657 ± 138 | 456 | 12.47 | 3303 ± 138 | 456 | 13.81 |
| Apr. 19 | 7600 ± 386 | 1280 | 16.84 | 4734 ± 257 | 852 | 18.00 | 2866 ± 271 | 898 | 31.33 |
| Apr. 26 | 7220 ± 522 | 1730 | 23.96 | 4341 ± 281 | 932 | 21.47 | 2879 ± 255 | 845 | 29.35 |
| May 3 | 8070 ± 401 | 1328 | 16.46 | 4355 ± 255 | 844 | 19.38 | 3715 ± 172 | 569 | 15.32 |
| May 10 | 7100 ± 327 | 1085 | 15.28 | 3837 ± 274 | 908 | 23.66 | 3263 ± 127 | 422 | 12.93 |
| May 17 | 7760 ± 402 | 1333 | 17.18 | 4350 ± 339 | 1123 | 25.82 | 3410 ± 159 | 528 | 15.48 |
| May 24 | 7130 ± 317 | 1052 | 14.75 | 4364 ± 275 | 913 | 20.92 | 2767 ± 217 | 718 | 25.95 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| May 31 | 7990 ± 657 | 2179 | 27.27 | 3865 ± 265 | 877 | 22.69 | 4125 ± 570 | 1888 | 45.77 |
| June 7 | 7510 ± 150 | 497 | 6.62 | 3717 ± 271 | 897 | 24.13 | 3793 ± 144 | 477 | 12.58 |
| June 14 | 8560 ± 461 | 1528 | 17.85 | 4624 ± 297 | 986 | 21.32 | 3936 ± 194 | 642 | 16.31 |
| June 21 | 8060 ± 460 | 1526 | 18.93 | 4095 ± 264 | 876 | 21.39 | 3965 ± 237 | 785 | 19.80 |
| Mean | 6761 ± 177 | 1334 | 19.73 | 3681 ± 97 | 730 | 19.83 | 3087 ± 95 | 718 | 23.26 |
| Minimum | 4110 | | | 2369 | | | 1679 | | |
| Maximum | 8560 | | | 4734 | | | 3997 | | |




TEXT-FIG. 1. Mean values for consecutive total white blood, granular and non-granular cell determinations as percentage deviations from standard values. 1927–28.




TEXT-FIG. 2. Mean values for consecutive total white blood, granular and non-granular cell determinations as percentage deviations from standard values. 1928–29.

in Text-figs. 1 and 2. The results have further been considered from the standpoint of the total granular and the total non-granular cells (Tables I to V; Text-figs. 1 and 2). In all cases, the observations are presented in the form of weekly group means together with the probable errors of the means, the standard deviations, and the coefficients of variation. The series of curves in Text-figs. 1 and 2 representing the smoothed means are drawn in the form of the percentage deviations of these values from standard values (1, 2). The standard values used are as follows: Total white cells, 9560 per cubic millimeter; total granular cells, 5505 per cubic millimeter; total non-granular cells, 4050 per cubic millimeter. Other curves illustrate the smoothed values of the coefficients of variation of the group means.

## DISCUSSION AND SUMMARY

In discussing the results on the consecutive total white cell counts of 5 groups of normal rabbits studied over long periods of time, it will be convenient to consider the observations of each group in chronological order. The results will then be taken up from the standpoint of the granular and the non-granular cells.

Group I was examined from October 24, 1927 to June 19, 1928. During the 9 months of the experiment, it was found that the mean total white cell counts were considerably increased as determined by weekly observations (Table I). The character and extent of this change is shown in the curve (Text-fig. 1) which represents the percentage deviations of the smoothed weekly means from a standard value of 9560 cells per cubic millimeter.

From an initial level of 10 per cent below the standard value in October and November, 1927, there was a fairly regular increase to 25 per cent above it by the end of February; in March and April, somewhat lower levels were observed, but in May there was another rise to 35 per cent, followed in June by slightly lower counts. It should be noted that the curve of the smoothed coefficients of variation of the means (Text-fig. 1) shows only slight irregularities throughout its course, thus indicating that the alterations of the mean values were generally characteristic of the group as a whole.

In the case of Group II (Table II) which was examined from March 29 to June 19, 1928, a similar increase of the total white cells was observed.

The level of mean values was lower than that of Group I for the same period, but the general character of the increase resembled in a striking manner that of the former group (Text-fig. 1). The curve of the smoothed coefficients of variation of the weekly means of Group II is quite uniform and occupies the same level as that of Group I.

The mean white cell values of Group III, followed from September 20 to November 22, 1928 (Table III) were lower than those of Group I or of Group II and in addition, differed from these first groups in showing only minor fluctuations.

The curve illustrating these findings (Text-fig. 2) presents few irregularities and is, in fact, almost a straight line at the 20 to 25 per cent level below the standard value. The first part of the curve of the smoothed coefficients of variation of the weekly means (Text-fig. 2) is slightly higher than those of Groups I and II but the second half is entirely comparable.

The fourth group of rabbits was followed from November 27, 1928 to June 18, 1929 (Table IV). In general, the level of the white cell means (Text-fig. 2) was similar to that of Group III and lower than that of Group I examined the previous year. It was found, as was the case with Groups I and II, that these values became considerably increased.

In November and December, the lowest counts of the group were observed; in January and February, higher values prevailed; during March and April, slightly lower levels were fairly well maintained with a second increase at the end of April which in turn, was followed by a steady decline to values comparable to those observed at the beginning of the experiment. The coefficients of variation of the white cell means of Group IV were of the same general order of magnitude as those of the first 3 groups, but they were somewhat more irregular as is brought out by a comparison of the respective curves of the smoothed values shown in Text-figs. 1 and 2.

In comparing these changes with those of Group I observed during a similar period of the previous year (Text-figs. 1 and 2), it will be noted that as far as form and direction are concerned, the curves for these groups bear a general resemblance to each other up to May and June. In the case of Group I, higher levels continued to be maintained during these months although the final direction of the curve is downward; in the case of Group IV, the downward trend which began in April was continued throughout June.

weeks respectively, the values during the latter part of the experiments were all higher than those of the first weeks. Both the granular and non-granular cell means participated in this change, but from a relative standpoint, the latter values showed the greater alteration. In the case of Group IV, followed 29 weeks, a similar increase of the total white cells occurred although it was not sustained during the last 8 weeks; the granular cell means of this group showed a greater relative change than those of the non-granular cells.

In connection with these findings of augmented white cell counts over periods of several months, it might be thought that the increasing age of the rabbits was a factor of some significance; at the beginning of these experiments, the animals were considered to be 6 to 8 months old. According to the recent observations of Cheng (5), however, the numbers of leucocytes in the blood stream of rabbits 9 to 16 months of age, although fluctuating at a high level, are not increased. The question of intercurrent infections such as snuffles, as a factor in the production of increased white counts will be considered in connection with the results on the individual classes of white cells (3, 4).

The significance to be attached to the major changes in the levels of the total white, the granular, and the non-granular cell means over periods of time is indicated by the following examples of the ratios of the differences of various mean values to their probable errors.

| Group Number | Total white cells | | | Granular cells | | | Non-granular cells | | |
|---|---|---|---|---|---|---|---|---|---|
| | Dates of mean values | | Ratio | Dates of mean values | | Ratio | Dates of mean values | | Ratio |
| I | Oct. 24 | June 19 | 2.85 | Oct. 24 | June 19 | 2.47 | Oct. 24 | June 19 | 2.64 |
| | Mar. 13 | June 5 | 4.36 | Dec. 6 | Feb. 28 | 3.30 | Feb. 21 | Mar. 20 | 7.52 |
| II | Mar. 29 | June 19 | 7.01 | Mar. 29 | June 19 | 3.67 | Mar. 29 | June 19 | 6.61 |
| III | Sept. 20 | Nov. 16 | 2.00 | Sept. 20 | Nov. 22 | 3.60 | Sept. 20 | June 19 | 1.64 |
| IV | Nov. 27 | Jan. 2 | 4.55 | Nov. 27 | Feb. 19 | 8.33 | Nov. 27 | Jan. 2 | 5.20 |
| | Apr. 9 | June 18 | 5.39 | Feb. 19 | June 18 | 8.75 | Jan. 2 | Apr. 23 | 7.13 |
| V | Dec. 29 | June 21 | 4.82 | Dec. 29 | June 21 | 5.43 | Dec. 29 | Jan. 24 | 5.44 |
| | Jan. 24 | Apr. 5 | 8.76 | Jan. 4 | Apr. 5 | 7.09 | Jan. 24 | June 7 | 10.16 |

As was the case with the erythrocyte and hemoglobin mean values of these experiments (1), the general level of the total white cell means was higher in the groups of rabbits examined during 1927–28 than in those examined in 1928–29. This was also true of both the granular and the non-granular cells but was less pronounced in the former than in the latter class.

The present results are analogous to those of the erythrocytes and hemoglobin (1) in still another respect. It has been shown that as far as general direction or trend of the total white cell means is concerned and in the case of many individual fluctuations as well, two groups of rabbits examined during the same months behave in a similar manner. In the case of the granular and non-granular divisions of white cells, however, the resemblance is not always as striking. With the granular and non-granular cells of Groups I and II and with the granular cells of Groups IV and V, the similarity was quite pronounced, but with the non-granular cells of Groups IV and V, the parallelism was less marked.

In most cases, an alteration in the trend of the granular cell means of a group was accompanied by a movement of the non-granular cell values in the same direction but in certain instances, a change in the opposite direction was observed.

The period of most frequent point to point fluctuation of the total white cell and of the granular and non-granular cell means of the 4 groups examined for several months occurred in February, March, and April of both years (1928 and 1929). A similar but less pronounced phase of irregularity affecting chiefly the total white and the granular cells was observed in October and November of 1927 but not in the fall months of 1928.

## CONCLUSIONS

Consecutive weekly observations on the total white cell count of the peripheral blood were made on 5 groups of normal rabbits, a total of 45 animals, during a period of 20 months from October, 1927 to July, 1929. The duration of individual group examinations varied from 8 to 35 weeks.

In the case of 4 groups followed 13 to 35 weeks, the general trend of the total white cell means was toward increasing values; with the

group followed 8 weeks, the means were maintained at a constant level.

The changes in the levels of the granular cell means were usually accompanied by changes in a similar direction of the non-granular cell means. In the case of 3 of the 4 groups followed for the longest periods, the greatest relative alterations occurred in the non-granular cells.

The fluctuations in the mean values of the total white cells and of the granular and the non-granular cells which were observed in one group of rabbits, were also generally observed in another group examined during the same months.

The period of greatest irregularity in the mean values of the total white cell means and of the granular and the non-granular cell means as well, occurred during the late winter and spring months of both years.

The general level of the total white cell mean values in the groups examined during 1927–28 was higher than that of the groups observed during 1928–29. A similar difference was found in the granular and non-granular mean values but it was somewhat less marked in the case of the granular cells.

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# TOOTH GROWTH IN EXPERIMENTAL SCURVY

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Plates 1 to 4

(Received for publication, March 22, 1930)

The lesions in the incisor teeth of scorbutic guinea pigs have been repeatedly studied since their recognition by Jackson and Moore (1). This paper is concerned with an associated phenomenon, namely, the rate of tooth growth in scurvy and partial scorbutic states as well as the effect of stress upon growth.

Through the work of Zilva (2), lesions of the roots of the incisor teeth of guinea pigs have been established as the first expression of a deficiency of Vitamin C. Höjer utilized this fact in testing the antiscorbutic value of foodstuffs, the minimal protective dose being the smallest amount of the antiscorbutic which would completely protect the teeth of guinea pigs throughout a 20 day period (3). As Eddy (4) has shown, the minimal protective dose by this method is much greater than by the more generally employed test (the Sherman method), which employs the weight curve of the animal fortified by certain clinical observations as pain over joints, presence of hemorrhages, etc. These and other related facts have been recently emphasized by Hanke (5), and others, and the rôle of Vitamin C in tooth development and health has become a subject of considerable interest.

## *Rate of Tooth Growth*

We have found one reference to the influence of diet on rate of tooth growth. Orban (6) noted the rate of growth of rats' teeth when starch alone was fed and found it less rapid than when wheat, meat and greens were given. Feeding wheat and cod liver oil, the growth rate obtained approximated the rate secured with wheat, meat and greens. The addition of lemon juice did not increase this rate.

In our studies rate of growth was measured by clipping the exposed portion of one of the lower incisor teeth every fifth day. A pocket finger nail clip was

found to be the most satisfactory instrument for the purpose since it was of convenient size, preserved the whole of the fragment intact, and required no prying force, hence producing the minimum of trauma to the root of the tooth. If, after repeated clipping, the tooth became very fragile and brittle it was first cut three-fourths through with a very thin carborundum disk used on a dental engine, and then clipped. By folding the pig's ears forward and keeping the disk wet the operation does not excite the animals. The length of the fragment was measured with vernier calipers. Results have been expressed as averages in millimeters per day. The animals used in the experiment were observed for periods of varying length, the shortest being 20 days and the longest period 90 days.

Since the normal rate of tooth growth was unknown, this was first established, using animals from 4 to 10 weeks of age, and from 110 gm. to 600 gm. In the smaller animals the rate of tooth growth was slightly less rapid than in the larger, but this factor was too small to affect the result of the feeding experiments.

The basal diet used was the Sherman-La Mer (7) to which 1 per cent of cod liver oil had been added. During the period of test the animals were scored by the Sherman method for degree of scurvy and were weighed every fifth day. At the end of the experiment the animals were killed and dissected. The lower jaws, ribs and extremities were removed, fixed, and studied histologically. The incisor roots were sectioned at various levels, one section regularly being removed at the level of the first molar for comparison with Höjer's standards for the estimation of degree of scurvy.

Table 1 summarizes the results obtained by varying amounts of orange juice, tomato juice and turnip greens as the sole source of Vitamin C in combination with Sherman-La Mer basal diet. The group on green vegetables represents the maximum rate of tooth growth and the animals were free of scurvy.[1] In this group the rate of tooth growth was 0.850 mm. daily in contrast to the minimal rate in the control animals of 0.306 mm. daily. In every series the increase in Vitamin C source stimulated growth rate.

Examination of the animals on these diets revealed certain collateral data of value which can be summarized as follows:

1. Animals given basal diet plus 5 cc. of orange juice daily, showed a rate of tooth growth of 0.750 $\pm$ 0.002 mm. per day. Slight but definite scorbutic lesions were present in the incisor roots though the long bones and ribs were free of scorbutic changes. The Sherman score showed no scurvy. 5 cc. was therefore not quite adequate for complete protection. 1 cc. of orange juice produced a growth rate of

[1] Greens are notably richer in Vitamin A than fruit juices and it is possible the high maximum with greens may owe something to this factor.

TABLE 1

*The Effect of Variation in Amount of Vitamin C Source on Guinea Pig Tooth Regeneration*

| | Vitamin source | Amount fed daily | No. animals | Days on experiment | Tooth growth mm. per day | Weight gains | Diagnosis by Sherman score | Diagnosis by Höjer method |
|---|---|---|---|---|---|---|---|---|
| A | Controls | Basal diet only | 10 | 20 days | 0.306±.0003 | −58 gm. | Acute scurvy | |
| B | Orange juice | 1 cc. | 3 | 20 " | 0.450±.002 | +40 " | Mild scurvy | 0.3 |
| | " " | 5 " | 6 | 20 " | 0.750±.002 | +55 " | No scurvy | 0.9 |
| | " " | 0.5 " | 3 | 90 " | 0.350±.002 | +180 " | Moderate scurvy | |
| | " " | 1 " | 2 | 90 " | 0.422±.002 | +230 " | Mild scurvy | |
| | " " | 5 " | 2 | 90 " | 0.750+.002 | +255 " | No scurvy | |
| C | Tomato juice | 2 gm. | 3 | 20 " | 0.505±.003 | +20 " | Moderate scurvy | 0.3–0.8** |
| | " " | 4 " | 3 | 20 " | 0.580±.0006 | +55 " | No scurvy | 0.3 |
| | " " | 8 " | 3 | 20 " | 0.623±.0006 | +70 " | No scurvy | 0.8 |
| | " " | 10 " | 6 | 20 " | 0.700±.0005 | +68 " | No scurvy | 0.8 |
| D | Turnip greens | 2 " | 2 | 20 " | 0.565±.0002 | +57 " | Mild scurvy | 0.3 |
| | " " | 4 " | 2 | 20 " | 0.640±.0002 | +72 " | No scurvy | 0.4 |
| | " " | 6 " | 2 | 20 " | 0.735±.0002 | +84 " | No scurvy | 0.8 |
| E | Mixed green vegetables* | 30 " | 3 | 20 " | 0.850±.002 | +75 " | No scurvy | 1.0 |

* Mixture included spinach, lettuce and carrots.

** An indefinite type of reaction.

$0.450 \pm 0.002$ mm. to $0.422 \pm 0.002$ mm. per day. Pronounced scurvy was found in the teeth but none in the long bones or costo-chondral junctions. The Sherman score was "mild scurvy."

Animals on 0.5 cc. of orange juice showed a rate of growth of $0.371 \pm 0.005$ mm. to $0.350 \pm 0.002$ mm. per day. Advanced scurvy was present in the teeth and slight changes in the ribs. The Sherman scoring was "moderate scurvy."

Similar results were secured with graded doses of tomato juice and turnip greens. The results are to be found in the table.

2. Animals given basal diet alone showed an average rate of tooth growth of $0.306 \pm 0.0003$ mm. per day during the first 15 to 20 days but at the end of that period growth ceased completely. However, after complete cessation of growth, the administration of 5 cc. of orange juice daily (commencing on the twentieth day) after a period of lag of 5 days, restored practically the normal rate of tooth growth. Also, so long as the diet afforded growth factor the rate remained constant for any given diet whether the period was 20 or 90 days.

### *Influence of Stress on Scorbutic Lesions*

In an earlier description of the changes encountered in the skeletal muscles of scorbutic guinea pigs (8), it was shown by one of the authors of this paper that exercise and stress determined largely the location and degree of the lesions. It was natural, therefore, to subject the teeth to similar tests since the anatomical lesions of scurvy probably all have this factor in common. As an amputated tooth would be subject to less stress in gnawing than its fellow of normal length, a comparison of the roots of the two teeth should be significant. For this reason in the animals of the series reported in Table 1, and in a number of others in which one incisor had been amputated, a comparison of the histological changes was made by preparing sections through both teeth just anterior to the separation of the mandibles.

The pulp of the incisors of guinea pigs is persistent, and growth, therefore, continuous throughout life. The pulp is roughly shaped like an elongated cone and covered with a surface of tall, slender and parallel odontoblasts (Fig. 2). Growth occurs through the elaboration, on the surface of this cone, of dentin, and, where exposed, enamel.

If a pig is deprived of antiscorbutic substance changes occur in the pulp. By the fifth day of complete deprivation the odontoblasts become shorter and blunter and lose their regularity of parallelism (Fig. 4). In place of dentin they form a semisolid material (Fig. 6) easily converted into dentin within a day by giving antiscorbutic substances, a phase carefully demonstrated by Wolbach and Howe (9).

If complete deprivation persists, the odontoblasts continue to regress and we have seen in the third week root pulp in which the parenchymal cells have left the dentin, developed into spindle and stellate shapes, and resembled fibroblasts (Fig. 5). Wolbach and Howe reported a similar change in the long bones. Death occurs regularly at this time and later stages have not been seen.

If the diet contains even small amounts of Vitamin C, a complete regression of odontoblasts does not occur and instead of fibroblasts the cells come to resemble osteoblasts, forming an intercellular matrix similar to bone which develops within the pulp until, in late stages, the entire cavity becomes filled with this so-called "osteodentin."

If the tooth has been clipped, the evidence of scurvy, both in amount of "osteodentin" and in the character of the cells will be less pronounced than in its fellow (Fig. 1). This has been consistently true of all the cases examined. In animals on a deficient diet for very short periods the amputated tusk may show a root of nearly normal appearance at a time when scurvy is evident in its fellow.

## DISCUSSION

These findings and the evidence of the influence of stress on the lesions, indicate that the scorbutic process is characterized by an inability of certain highly differentiated cells to form the intercellular substances natural to them. In cases of partial deficiency an inferior, substitute material may be formed, less highly differentiated than dentin (bone), but when deprivation is complete the cells dedifferentiate still further and form a still more primitive tissue, namely, fibrous tissue. This is similar to what occurs in the long bones and costo-chondral junction where the osteoblasts, appearing unable to form bone matrix, become fibroblasts.

The reaction is increased by demands made on the structure and the lesion constitutes a faulty reaction to stress in the absence of a necessary material.

## CONCLUSIONS

1. The incisor teeth of guinea pigs have a constant rate of growth in health.

2. Deprivation of Vitamin C causes the teeth to cease growing. Readministration of the vitamin restores the growth.

3. Administration of small amounts of antiscorbutic substance results in rates of growth roughly proportional to dosage.

4. Under standard experimental conditions used in the testing of foodstuffs for antiscorbutic value, the rate of tooth growth would appear to be a precise indication of the degree of scurvy, being more delicate than the Sherman score, and more constant as well as more simple, than the Höjer method.

5. Stress in terms of usage appears to exaggerate the scorbutic lesions in the teeth.

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## EXPLANATION OF PLATES

### PLATE 1

FIG. 1. Low power microphotograph of incisor teeth showing differences in structure between amputated (A) and natural (N) teeth in scurvy. The line of odontoblasts (0) is heavier and is formed of longer, more parallel cells in the amputated tooth and the imperfect dentin (N) is narrower.

The difference in breadth of the original dentin is due to the more rapid growth of the amputated tooth and is not related to the scorbutic process.

### PLATE 2

FIG. 2. Highly magnified odontoblast line in normal guinea pig tooth. The odontoblasts are slender, parallel and long. The dentin canals are clearly shown.

FIG. 3. Same magnification as Fig. 2. The animal had been given basal diet

plus 3 cc. orange juice daily for 20 days. The cells are shorter and less regular. Near four o'clock an island of imperfect dentin is being formed within the pulp itself by migrated odontoblasts.

PLATE 3

Magnifications same as in Figs. 2 and 3.

FIG. 4. Line of odontoblasts after 5 days on a diet completely deficient in antiscorbutic substance.

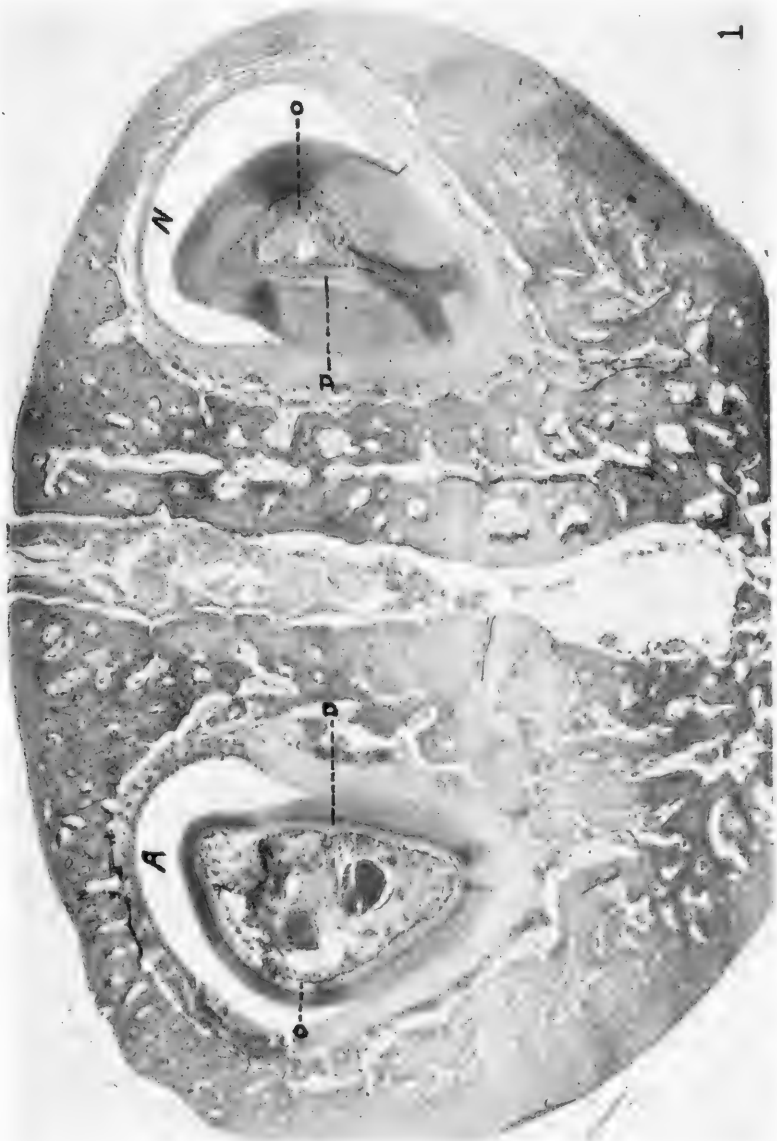
FIG. 5. In this animal, which had been for 21 days without antiscorbutic substance the odontoblasts have lost their natural appearance and resemble fibroblasts.

PLATE 4

Magnifications same as in Figs. 2 to 5.

FIG. 6. Illustration of recovery of function by odontoblasts. The animal had been on a Vitamin C-free diet for 10 days and was then given orange juice for 5 days. The cells have become more orderly and a zone of fresh pre-dentin has been formed.

FIG. 7. An animal on basal diet alone for 20 days. The odontoblasts seem to have disappeared.




(Dalldorf and Zall: Tooth growth in experimental scurvy)

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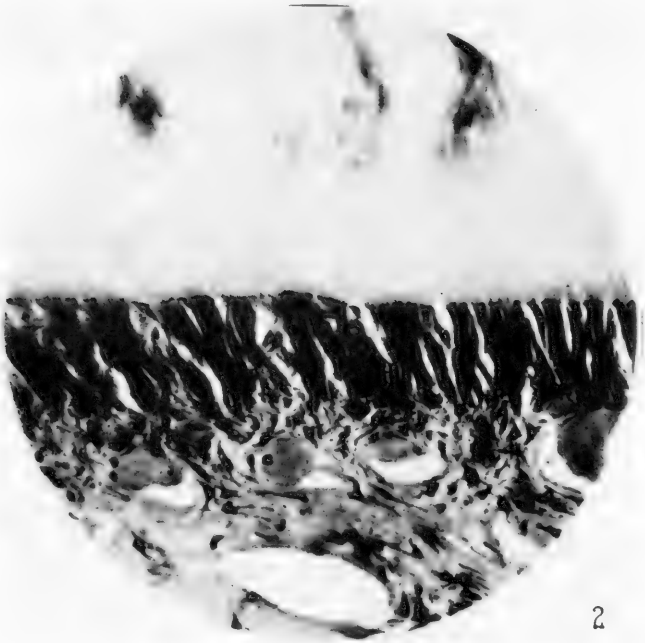

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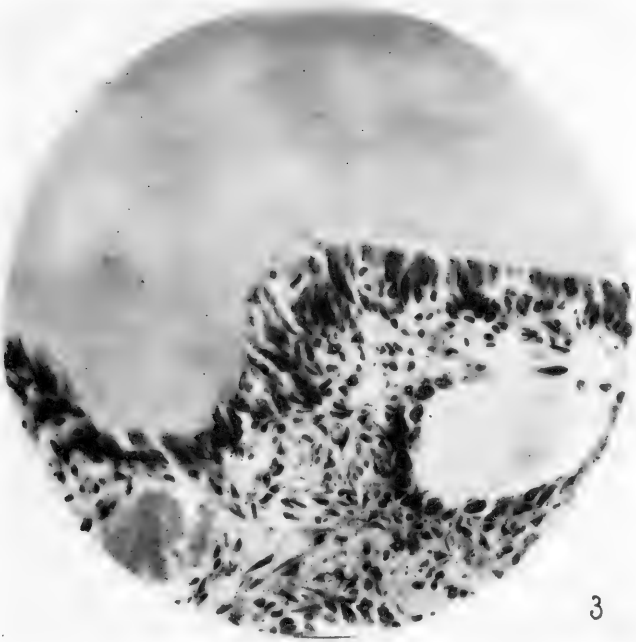

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(Dalldorf and Zall: Tooth growth in experimental scurvy)

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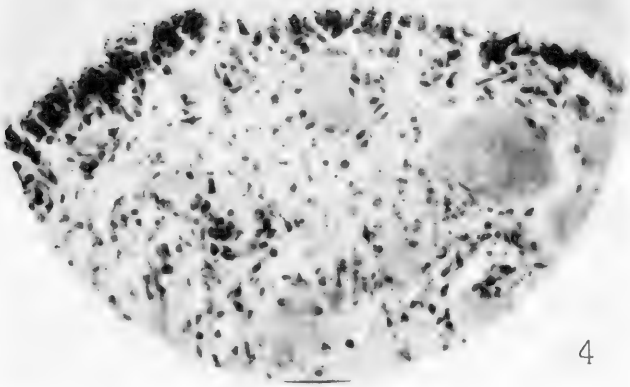

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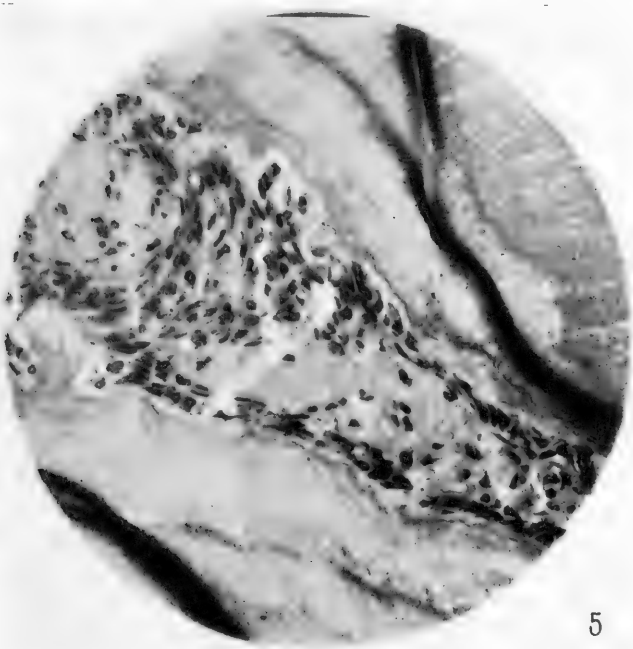

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(Dalldorf and Zall: Tooth growth in experimental scurvy)

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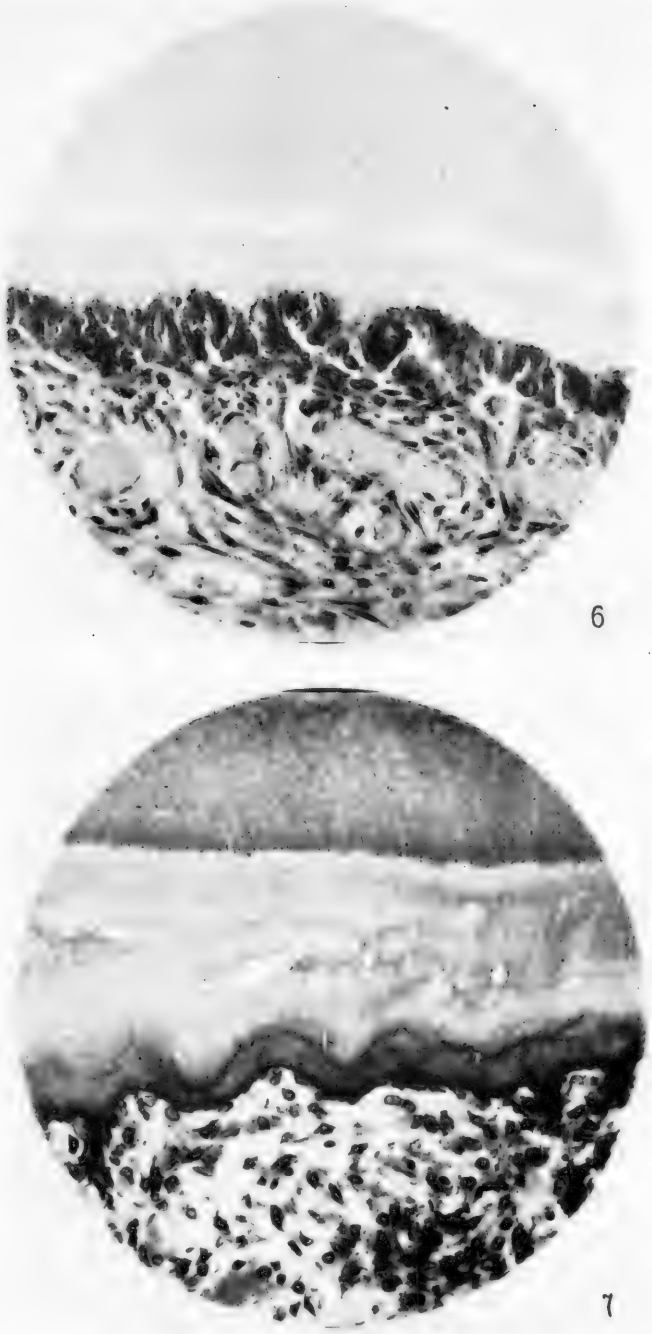


(Dalldorf and Zall: Tooth growth in experimental scurvy)

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# STUDIES ON THE BLOOD VESSELS IN THE MEMBRANES OF CHICK EMBRYOS

## PART I. ABSENCE OF NERVES IN THE VASCULAR MEMBRANE

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PLATE 5

(Received for publication, April 23, 1930)

Experiments on the behavior of the blood vessels have with few exceptions been confined until now to tissue in which there are nerves. In all the organs of the body in adult human beings or in animals that have attained full growth, in which such investigations have taken place, the presence of vasomotor nerves has been demonstrated either anatomically or physiologically (1).

It is to be expected that in investigations of the reaction of blood vessels in tissues or an organ which is not innervated, the part usually played by the nervous factor can be ascertained. Experiments carried on in such preparations might make possible in a new way the elucidation of questions having clinical interest, as for instance, those arising in arterial hypertension (2) in which hypersensitivity of all vessels is present as well as in arteriosclerosis (3) in which the level of irritability is reduced and finally, in cases of inflammation (4, 5) in which the part played by the nervous system is still the subject of discussion.

### *On the Absence of Nerves in the Yolk-Sack of Chick Embryos*

Concerning the occurrence of nerves in the membranes of chick embryos there exist in the literature, so far as we know, no references (6). There are, however, numerous investigations on the innervation of membranes of human beings and other mammals to which we intend to refer by way of comparison. The question whether the umbilical cord and the placenta contain nerves dates according to Schott (7) from the time of Galen. Reports of more recent investigations, to the

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literature of which Schmitt (8) has referred, and which are based in part on histological investigation carried out in tissues studied mainly by the method of impregnation with silver, and in part on pharmacological methods, contain the conclusion that the umbilical cord contains nerves of its own but only in that portion close to the body, and that its greater distal portion as well as the placenta are free. Ikeda (9) recently, at the suggestion of Aschoff, has reinvestigated this matter and has confirmed the statements of earlier authors.

We have attempted to solve the question as to whether the membranes of chick embryos contain nerves in part by physiological, in part by histological investigations.

### *Physiological Experiments*

*Technique.*—The experiments were conducted in a constant temperature chamber at 38°C. Sources of error due to fluctuation of temperature are therefore avoided. Eggs in all stages of development, but mostly those 3 to 4 days old, were studied. In older eggs the separation of the membranes is more difficult but can, after practice, be conveniently managed. Eggs were opened at the air-chamber end without turning or jarring them, as much of the shell as was necessary to expose the vascular region being removed by means of forceps. The embryo surrounded by sufficient of its membranes could by this means be exposed. The loss of albumen was, of course, prevented. The manipulation of the egg on opening it must be gentle; otherwise the blood vessels will be found even at the beginning of the experiment to be contracted, so that weak stimuli would lead, while they are in this state, to erroneous inferences.

For electrical stimulation the usual form of a reliable inductorium was used. The source of current was an Edison storage battery. When drugs were injected the point of the needle was inserted laterally until it came to lie under the membranes, since preliminary experiment showed that better and more uniform absorption took place than if these substances were applied drop-wise to the surface of the preparation. The experiments were brief, never lasting longer than 10 minutes so that errors due to desiccation might be avoided. The observations were made with a Zeiss dissecting microscope furnished both with binoculars and bin-objectives. The embryos were protected from excess heat by placing a water filter between the source of light and themselves.

We observed the behavior of blood vessels of the yolk membrane in some 1600 eggs at various ages. The experiments which we report now, selected from the great number which were performed, permit our making the following statements concerning the innervation of these vessels.

1. One locus of the membrane was touched several times with a fine

glass rod. Depending on the number of contacts and their strength, either constriction or dilatation took place. These stimuli brought about this effect both in capillaries and in small arteries. Even the strongest stimulus of this mechanical sort brought about no change in the immediate environment. The effect of the stimulus was strictly confined, wherever it was exercised, to the site which was stimulated.

2. A faradic current was employed to stimulate a given locus of the vascular layer, electrodes of varying diameters being used. Both the smallest arteries and the larger arterial branches were stimulated. One electrode consisted of cotton wool moistened with Ringer's solution which was placed on the membranes at a distance from the vascular zone, that is to say, beyond the marginal vein. The other electrode had the diameter of a hair. As the result of the stimulus, dilatation occurs either in a capillary or at one point of the artery, but is so small in extent as to be recognized only microscopically. If an electrode having a larger diameter is used the effect is apparent in a larger area. In all cases the effect of stimulation, whether it be contraction or dilatation, is to be recognized only at the point at which the stimulus is applied or, when the strongest current in our scale was used, in the immediate neighborhood. Involvement of neighboring regions or a propagation from the capillaries to larger arteries, or propagation in the reverse direction was never noticed.

The following experiment was made with the view to extending these observations and to detecting the possible existence of conduction over nerve paths:

The central end of an artery which led to a vascular area which we wished to stimulate was tied off and the whole vascular region was isolated by a circular incision so that every connection with the embryo was severed. In this isolated vascular region electrical stimuli were made just as before. The result of stimulation was the same as when the region was still in continuity with the embryo. On account of the absence of a circulation, that is to say of additional blood, the column of blood in the vessel when it was dilated, the degree of dilatation depending, of course, on the strength of the stimulus, appeared to be paler than before. Measurement of the diameter microscopically indicated, however, the same degree of dilatation as when the connection with the embryo was intact.

3. A fine hollow needle was inserted outside the vascular area and then pushed forward so that its opening came to lie beneath it.

Sodium iodide solution 6 per cent was injected in the amount of 0.1 cc. Dilatation of the vessels occurred at once but only at the point of injection. If larger amounts were injected a larger region was affected. If the same amount of saturated solution of sodium iodide is injected, stasis in the region of the drop immediately occurs. On account of the stagnation of blood which results, blood accumulates in the portion of the vessel central to this point. This appearance quite obviously represents a secondary phenomenon. The effect of the stimulus itself remains confined to the site of the stimulus. It appears to be unnecessary to report our experiments with other chemicals. The result was always the same—the stimulus affected merely the locus at which it was applied. If larger amounts were injected there was always the possibility that after absorption of the substance, and distribution in the circulation, a result would occur at points far removed from the point of stimulation and would there give the impression that a stimulus had been applied. But when small amounts are used and are confined to a small area the effect of the stimulus takes place only at the point of stimulation.

These observations differ from those of blood vessels which are known to be innervated. In the skin there occurs with every stimulus of low intensity an easily demonstrated erythema, an effect of the stimulus which can be seen far beyond the point of stimulation (Ebbecke (10), Lewis (11)). Reactions like this can be understood only as the result of an action in which the nervous pathways are involved. In the pancreas of rabbits for instance, Ricker and Regendanz (4) found that the effect of strong stimuli involved a larger region than the point stimulated in contrast to that of weaker ones, the effect of which was merely local. In such cases the result is brought about by a reflex (nervous) action. In our experiments we could never bring about an effect beyond the region of stimulation even if strong currents were used. The restriction of the reaction of the blood vessels in the vascular areas in our preparation to the site of the stimulus suggests strongly that there are no nerves in the vascular layer of these embryos.

Among the drugs that have been tested which have an effect upon the blood vessels adrenalin is the only one which has an action conspicuously different from the ordinary on the vascular area of these membranes.

Adrenalin, in a solution of 1 per mille, was injected through a needle at a point just under the vascular area since it had been learned that adrenalin applied to the surface had no effect even when large amounts were used. 0.1 cc. of a 1 per mille solution brought about no visible contraction. It was not until an amount of 0.4 cc. or more had been injected that a contraction of the blood vessels took place, but even then the effect was never immediate. Indeed the earliest evidence of action was observed only after ½ minute. In most experiments contraction did not begin until the end of 4 to 5 minutes.

But even large doses like those just mentioned were not effectual in all cases. Of 38 observations, constriction took place in 25, in 4 the effect was uncertain, and in 9 there was no effect at all. Because of the weak action of the drug we were obliged to use relatively speaking unusually large amounts of fluid, 0.5 cc. or more. Control experiments with equally large doses with physiological salt solution of about the same hydrogen ion concentration, had as a matter of fact a very similar result. We are inclined to attribute the effect to the solvent therefore rather than to adrenalin. Even if we were to attribute the effect observed to adrenalin, the extent of it was remarkably small.

The contrast between this result and that in tissues which are normally innervated is striking. For in the latter it is easy to obtain a reaction everywhere by the local application of 0.1 cc. of a 1 per cent solution. The small effect in the vascular area of our preparations is accordingly altogether unusual.

Results similar to ours have been reported by Schmitt (8) in the case of blood vessels of the placenta which were treated by a perfusion method. If into the blood stream of the perfused placenta 1 cc. of suprarenin in the concentration of 1 to 1000 was injected, no effect was observed on the rate of flow in the vessels. Since it is generally recognized that the action of adrenalin takes place by virtue of its effect on nerves or on mechanisms of a nervous nature, the small effect of adrenalin on the vessels of the vascular membrane of chick embryos may be taken to represent further evidence that a nervous mechanism is wanting in these structures.

### *Anatomical Evidence*

We have examined the embryonic membranes of chicks by means of a large variety of histological methods using the mesentery of rabbits as well as other organs, amongst them the skin, heart and lungs, by way of controls. Our best results were obtained by the use of Bielschowsky's method of silver impregnation in block, and also with the rongalite white method.

*1. Investigations with Bielschowsky's Method.*—We impregnated by Bielschowsky's method, 6 embryos and their membranes aged 3, 4 and 7 days, according to the recommendations of Schmorl (12), and cut them in serial sections. In the case of older embryos only single sections of the membranes were made. As early as 72 hours after the beginning of incubation, small, fine nerves were found in the region of the brain and in the ganglia of the head. At the end of 96 hours nerves could be found everywhere in the brain and spinal cord as well as in the spinal ganglia (Fig. 1). At the end of 7 days nerves were to be found in all parts of the embryo.

Contrary to the situation in the embryo itself, nerves were to be found nowhere in the membranes. In those sections in which the transition from embryo to membranes could be clearly seen it was apparent that the nerves were confined to the structure of the embryo and did not proceed over to the membranes, as for example in a specimen 7 days old (Fig. 2, b). Small single fibrils were seen stretching beyond the region of the lateral limiting sulcus (Fig 2, a) but in no section could they be traced to the membranes themselves. The last of the nerves found in Fig. 2 is shown by means of greater magnification in Fig. 3.

*2. Investigations with the Rongalite White Method.*—Whereas the silver impregnation method according to Schmorl's description gave always completely satisfactory results, difficulties were encountered at first with the rongalite white method. The only investigators who seem to have used this method have been Kreibich(13) and Glaser (14, 15). They succeeded even in cases where other methods failed in obtaining first-rate results both with intra- and supra-vital staining. Since an intra-vital technique was impossible on account of the small size of the objects, we have used the supra-vital method only. The mesenteries of rabbits were again used as controls. Since both the authors just mentioned have described the method in a cursory way only, we have decided to describe it in detail, according to the procedure which gave us the best results.

Rongalite white, which is methylene blue reduced by means of rongalite, was prepared in the following way: 1 gm. of pure methylene blue was dissolved in 200 gm. of distilled water, was heated, and then 6 gm. of rongalite and 15 drops of hydrochloric acid were added. This solution was boiled until it became clear and was then filtered. Staining was carried out in the following way. Organs were taken from the embryo immediately after death and were bathed in physiological salt solution. Rongalite white 5 per cent to 10 per cent was next added. After pieces of tissue had been stained for 45 minutes they were fixed in ammonium molybdate 5 per cent. After thorough washing they were placed in absolute alcohol and xylol and were then drawn up on to glass slides.

By this method we examined the membranes of 19 embryos of 3, 4, 8, 9, 10, 13, 18 and 20 days' incubation after they had been stained and a control provided for each membrane. Whereas in the mesentery, nerves could be beautifully demonstrated everywhere, none could be found in any of the membranes.

## DISCUSSION

It appears then from our histological investigations that we were unable by means of the two best methods available to us to detect nerves in the membranes of the chick embryo. Neither did we find primitive cell processes which might be regarded as undifferentiated nerves. Our results therefore are in complete agreement with those which have been published in the case of the placenta both of man and of other mammals.

Is the conclusion to be drawn that the membranes contain no nerves? The objection may be made that since we are dealing with embryonal tissue we should not expect to find fully differentiated nerves. But the fact is that these tissues pass through a definite cycle of development, the course of which we have been able to follow from beginning to end, and that we have been unable to demonstrate nerves in any stage of their development, although the embryos themselves, from on the 3rd day on, always contained them.

Our anatomical results are in agreement with our physiological investigations. The absence of a reaction in the immediate neighborhood of an area, even when strong stimulation has been applied to the area itself, and the atypical behavior of the blood vessels in adrenalin experiments, can be understood only as resulting from the absence of nerves.

## CONCLUSION

The agreement of physiological experiment with anatomical findings justifies our conclusion: the blood vessels of the vascular membrane of chick embryos do not contain nerves.

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## EXPLANATION OF PLATE 5

FIG. 1. Chick embryo No. 177. Incubation period 4 days. Neurofibrils in a ganglion-anlage of the body. Silver impregnation. × 450.

FIG. 2. Chick embryo No. 173. Incubation period 7 days. A cross section through the body is shown at the point of transition from the embryo to the membranes. At (a) the lateral limiting sulcus appears, and at (b) the amnion and chorion. The neurofibrils farthest away from the body (Fig. 3) were found at (c). At (d) there is a cross section of an extremity. Silver impregnation. × 40.

FIG. 3. The region (c) indicated in Fig. 2 is shown. × 1000. It exhibits the most distant peripheral nerve found in this section.

(Lange *et al.*: Blood vessels in chick embryos. I)

# STUDIES ON THE BLOOD VESSELS IN THE MEMBRANES OF CHICK EMBRYOS

## Part II. Reactions of the Blood Vessels in the Vascular Membranes

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In recent years there has been lively discussion on the behavior of the vascular system especially concerning the dependence of reactions of the vessels on the nervous system (1, 2, 3, 4). In order to advance knowledge of this matter and to make clear what actually takes place, and to attempt to indicate the significance of these processes, it has become necessary to enlarge this study by examining a new and a greater variety of material. The blood vessels of the vascular membranes of chick embryos seem especially adapted to the ends in view. Membranes containing the vessels can be easily examined both microscopically and macroscopically after removing the shell and the shell membrane. Stimuli of any desirable kind may then be easily applied. Since it has been shown (Part I) that these tissues contain no nerves, the course of the reaction takes on a special interest.

### *A. The Effect of Electrical Stimuli of Varying Strength on the Blood Vessels*

*Faradic Stimulation.*—The technique used was the same as that employed in Part I. The electrode consisted of two copper wires each a millimeter thick and separated a distance of 1 mm. by means of collodion. The points of the electrodes, which alone came in contact with the tissue, were polished together with the collodion in which they were embedded, by means of sandpaper. Eggs were used after having been incubated 4 days.

In order to facilitate comparison, stimuli were made in the following experiments always at the same place, namely, the final pre-

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capillary branches of the artery which passes upwards towards the head of the embryo and to the right.

*Experiment 1.*—A very weak faradic current just strong enough to create a sensation of prickling on the moistened lips was used. After the electrodes had been placed, without exerting pressure, upon the appropriate vessels, current was allowed to flow through the electrodes for 10 seconds. They were then removed for 10 seconds in order to observe the effect which had been created, and then were replaced for a further 10 seconds. The duration of the stimulation was measured with a stop-watch. After the stimulus had been applied for 20 seconds, dilatation of the vessels appeared. The flow of blood in them continued to be rapid. In the surrounding vessels there was no change. We designate this stage in which there is dilatation with rapid flow "the stage of fluxion," adopting Ricker's term. 1 minute after the end of stimulation, the original diameter and rate of flow had returned.

From this experiment we made the following inference: The weakest possible faradic current brings about a brief stage of fluxion.

*Experiment 2.*—In this case a faradic current of medium strength which causes merely a burning sense when applied to the lips was used. The site of the stimulus was the same as in Experiment 1. The duration of the stimulus was 20 seconds, during the course of which there was an interruption of 10 seconds. At the end of 20 seconds, contraction of the blood vessels developed. The rate of flow was rapid. If the stimulus was continued for 20 seconds longer, the blood vessels shut. In the capillaries just peripheral, the flow ceased, so that the blood in them was motionless. Others were empty. The larger arteries central to these appeared unchanged. The flow of blood just proximal to the point of contraction was rhythmical. 5 minutes after the end of the stimulus the original diameter of the vessel was reestablished and the rate of flow was as before.

We made the following inference: A faradic current of medium strength brings about contraction of that arterial branch which has been stimulated.

*Experiment 3.*—A faradic current which was just strong enough still to be borne when applied to the lips, and which caused an unpleasant sensation of burning was used in the same way and at the same site as in Experiments 1 and 2. After 20 seconds there appeared a spindle-shaped dilatation, the degree of dilatation being greater than in Experiment 1. The rate of flow was at first slow, and then stopped after a number of seconds at a point further out than the site stimulated. Central to this point the dilated vessel was filled with blood. The blood corpuscles agglutinated so that they were no longer individually identifiable. Adopting Ricker's term we designate this "the stage of stasis." Capillaries peripheral to the site of

stimulus were empty. Just central and just peripheral to that portion of the affected vessels which was dilated, but within the region of influence of the stimulus, the arterial branch was contracted; in consequence, the dilated portion appeared as distinctly spindle-shaped. The spindle-shaped dilatation and the contraction of that part of the vessel just central to it appeared to occur synchronously. The capillaries beyond, that is to say peripheral to the vessels so affected, were empty. Transition between the dark red region of stasis and that region beyond which appeared light owing to the absence of blood could be recognized macroscopically as a sharp line. Stasis persisted throughout the observation and could not be influenced by any new stimulus.

From these findings we have drawn the inference that a strong faradic current brings about stasis.

In order to ascertain whether and to what extent dilatation depends on the pressure of the inflowing blood stream, we carried out the following experiment.

We placed a pincette which shut off the flow of blood on a small artery. A stimulus of the same strength as that employed in Experiment 3 was then applied to the artery. Dilatation of the same degree took place. The blood in the dilated vessel was, however, a lighter red since it was distributed in a greater space. After 2 minutes the pincette was removed. The dilated vessel was thereupon filled with blood and the spindle appeared to be quite full.

There appears, therefore, to have taken place, genuine primary dilatation with filling as a secondary phenomenon. The dilatation may become so great and the spindle so filled with blood that in young specimens the total volume of blood contained in the embryos has been seen to collect in the dilated area. Under these circumstances the heart continued to beat but appeared as a mere shadow, seeing that it was empty of blood.

Under similar experimental conditions a weak faradic current brings about dilatation of the arterioles of the vascular membrane with increased rate of flow, the stage of fluxion, a current of medium strength, contraction to the point of closure, and a strong current stasis of the blood in a dilated vessel. It appears therefore that the reaction depends upon the strength of the current. It is necessary next to ascertain whether other stimuli have the same consequences.

### *B. The Effect of Various Other Stimuli*

(*a*) *Mechanical Stimuli.*—In this case the stimulus consisted of touching the locus with a blunt glass rod. The site of the stimulus was as before. Because of the greater size of the glass rod, small arteries as well as the capillaries were involved.

The *gentlest possible* contact was established 3 times in 1 second, the intervals between being naturally brief. As a result the small arteries and capillaries dilated, becoming larger than those in the neighborhood. The rate of flow in the dilated vessels was rapid. This is the stage of fluxion. *Stimuli of medium strength* were applied 3 times, at the same rate as before and to the same site but with somewhat greater pressure. The small arteries and capillaries now contracted and disappeared. The site which was stimulated appeared as a lighter spot, empty of blood. The vessels in the surrounding region were not visibly altered and exhibited a rapid flow. 5 minutes later the small arteries again filled from the side of the heart and 1 minute later still the general appearance was the same as before the application of the stimulus. *Strong stimuli* consisted in bringing the glass rod in contact with the vessels 3 times, using quick strong strokes. An enormous dilatation of the small arteries and capillaries took place at once in the region stimulated. Centrally and peripherally, waist-like contractions were observed. The capillaries which were related anatomically to the area, but which were outside the region affected by the stimulus, were empty. An hour afterwards no change had taken place, stasis still persisting.

We drew the following inference: Depending upon the strength of the stimulus, mechanical irritation brings about a stage either of fluxion or one of contraction or one of stasis.

(*b*) *Ammonia.*—The site of stimulation was as before. The stimulating substance was applied through the needle of a small syringe.

*Experiment 1.*—When 0.5 cc. of a 1 per cent solution was injected, dilatation took place at once as well as a rapid flow of blood at the site of stimulation.

*Experiment 2.*—10 minutes after the stage of fluxion had been brought about as in the first experiment, the injection of ammonia (0.5 cc.) was repeated. The capillaries and the smallest arteries contracted. When a third injection of the same amount was made there was moderate stasis in the capillaries whereas the small arteries were contracted.

*Experiment 3.*—When 0.1 cc. of a 5 per cent solution of ammonia in Ringer's solution was injected slowly, the arterial branch which was affected dilated enormously at once while complete cessation of blood flow through it took place. Dilatation was so complete that stasis did not develop as in the previous experiment but instead coagulation of the blood. Agglutinated cells floated about in a clear medium within the vessels.

We have drawn the following inference: Ammonia brings about the stage of fluxion, of contraction, or of stasis and hemorrhage, according to the concentration used and the period during which it acts. Strong concentrations cause coagulation (pseudostasis).

We have chosen the experiments described as examples of the effects of 3 qualitatively different kinds of stimuli. But we have also made experiments with mustard oil, with silver nitrate, with potassium iodide and with oil of camphor. In principle the action of all was the same. It was possible by grading the concentration and the dose, to bring about the same effects as when the faradic current, the glass rod or ammonia was employed. Since the stimuli used were able to produce an effect on blood vessels in tissue in which there were no nervous elements, we have been forced to conclude that they have their effect upon the cells themselves and that these have an inherent power to exhibit the motions which we have observed.

The action of adrenalin as reported in the first of these papers was inconstant. Atropin had no action at all even in concentrations as high as 1 per cent. Cold and heat had no distinct effect. For that reason we were unable to utilize different degrees of temperature as means of establishing grades of reaction on the part of the vessels.

In our experiments we have found then that with effective stimuli there is a systematic relation between the strength of the stimulus and the reaction of the blood vessels, in the sense described by Ricker in the case of blood vessels in which a nervous apparatus exists. A weak current as we have shown is followed by the stage of fluxion, one of medium strength by that of contraction and a strong one by stasis. The general rule of the relatedness of a particular form of reaction to a particular intensity of stimulus is exhibited also by the non-innervated blood vessels studied in these experiments and by the kinds of stimuli which were employed. Our observations therefore confirm Ricker's theory concerning the laws governing the relation between reactions and the strength of stimuli. In the case of blood vessels which are not innervated it appears that the strength of the stimuli not the nature of the stimulus constitutes the determining factor. The same forms of reaction were found whenever it was possible to grade the strength of the stimuli irrespective of their kind.

### *C. The Effect of a Given Stimulus upon Different Portions of the Vascular System*

In the experiments so far reported, stimuli were always permitted to exert their effect upon vessels of a like diameter. We have next to report our study of the effects of the same stimulus on capillaries, small arteries and on arterial main stems. For this purpose we have employed faradic current, mechanical stimulus, or one of the chemicals already mentioned, mustard oil, silver nitrate, potassium iodide or the oil of camphor.

*Experiment 1.*—We stimulated a capillary area with a very weak current for 20 seconds. There resulted the stage of fluxion. When the same stimulus was applied to small arteries or the main stems of arteries, no visible reaction took place and we drew the following inference: A stimulus which brings about the stage of fluxion in capillaries has no effect upon arteries.

In *Experiment 2* a stimulus of medium strength applied for 20 seconds to a small artery caused contraction. A stimulus of the same strength applied for the same length of time to the capillaries caused stasis. If the same stimulus was applied for the same length of time to an arterial stem, no distinct effect ensued although the appearance of a slight degree of dilatation occurred occasionally. From this experiment we draw the inference that a stimulus which causes contraction in small arteries causes stasis in the capillaries.

*Experiment 3.*—If a strong stimulus was applied for 20 seconds to an arterial stem, stasis took place in the vessel which had undergone spindle-shaped dilatation. The same stimulus if applied to small arteries or capillaries likewise caused stasis. And we draw the inference that a stimulus which causes stasis in arterial stems does so likewise in small arteries and capillaries.

SUMMARY

A comparison of the effect of stimulation upon the arterial subdivisions shows that these differ in irritability. The capillaries are most irritable, then come the small arterial branches, and last the main stems of arteries, these being the least irritable. The non-innervated blood vessels of the vascular membranes exhibit therefore the behavior described by G. Ricker in the case of those which have a nervous apparatus. We have demonstrated accordingly similar properties in vessels which are not innervated as in those which are. Certain differences exist however. The action of adrenalin on the vessels of the vascular membrane is inconstant and certain other sub-

stances are wholly ineffective. Ricker's experiments have advanced knowledge on the regularity of the vascular reactions and have paved the way for these experiments of ours. The result of our experiments with vessels free of nerves suggests that a reexamination may be fruitful of the mechanism which obtains in innervated vessels. There can be no doubt that nerves play a rôle in the behavior of vessels; it appears now to be necessary to define more accurately precisely what this is.

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# STUDIES ON THE BLOOD VESSELS IN THE MEMBRANES OF CHICK EMBRYOS

## Part III. Anatomy and Physiology of the Blood Vessels at Different Ages*

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The development of the vascular system of the membranes of chick embryos passes through a definite cycle. It can be followed from its inception in the blood islands, through the stages in which the blood vessels are finally developed, to the time when it is discarded at the time of hatching. It ought to be possible therefore to correlate changes in function with the anatomical changes. We hoped to obtain an insight into the alterations which take place in both, and to do this the more readily owing to the short duration of the whole cycle.

### *Physiological Experiments*

The irritability of the vessels was studied by applying stimuli of the same strength to them from their inception to the time of hatching. The experiments were carried out in eggs that had been opened as in Parts I and II of these studies.

It was necessary to introduce a slight modification in technique in the case of older embryos for in them it is more difficult to remove the membrane than in embryos 5 days old. The eggs were therefore kept on end, the air-chamber upwards, for 12 hours before the beginning of the experiment. With practise it became easy after opening the shell in the region of the air-chamber, to remove the shell membrane with fine forceps and scissors. The blood vessels then are as easy to approach as they are in younger embryos in which they are disposed along

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the long axis of the egg. The stimuli employed were a faradic current of known strength, sodium iodide, ammonia and mechanical stimulation with the use of a glass rod. As in our earlier experiments we ascertained which strength of stimulus it was necessary to employ in order to secure stasis in an egg after 3 days of incubation. We then chose a stimulus based on this, but weaker, maintaining all the stimuli which we employed at the same strength for all other ages. On account of the similarity of our observations it is necessary to describe only our results.

At the time when there are only blood islands and no regular flow of blood, contraction and dilatation do not occur. At this early age the occurrence of coagulation as the result of the use of chemicals in strong concentrations can naturally not be observed. Our observations begin therefore with the behavior of blood vessels at the time when a regular rate of flow begins, namely, on the 3rd day. All stimuli which bring about stasis in arteries of the membrane on the 3rd day of incubation produce the same dilatation in the same length of time in arteries of the same size and in the same way in all eggs of all later ages. The criterion of a strong stimulus has been described in previous sections of these papers and may be defined as follows: In the case of a faradic current it is one which on moist lips causes an unpleasant sensation of burning. In the case of sodium iodide a concentration of 6 per cent in the amount of 0.1 cc. and in the case of ammonia a 5 per cent solution of the same amount. We chose a weaker current, as has been said, one which in small arteries causes contraction and studied the reaction to it through all ages up to the 19th day, that is to say, shortly before hatching of the egg. In all stages of development the effect of stimulation of arteries of the same caliber is the same, namely, contraction.

A difference in the manner of reaction is always visible. Capillaries react to weaker stimuli than do arteries. In this sense specimens which are younger differ from older ones. In specimens 3 and 4 days old, all the vessels respond to stimuli which in older embryos affect only those vessels which have the same small dimensions as the young ones. The larger vessels in older embryos require stronger or longer stimuli. For example, stasis was observed in all arteries in 3 to 4 day old embryos as the result of applying an electrical stimulus of a certain strength for 20 seconds. In specimens 18 days old on the contrary this stimulus applied to large arteries required twice the dura-

tion, namely, 40 seconds or a correspondingly stronger stimulus for 20 seconds to bring about the same effect. This striking difference is to be explained in terms of histology.

*Anatomical Investigations*

There are satisfactory investigations by Popoff (1) and H. Virchow (2) on the distribution and branching of blood vessels of the vascular membrane of chick embryos at various ages, but a comparative histological study of these vessels at all stages of development has not so far been published. In especial nothing is known on the presence of elastic tissue nor of the appearance of degeneration in the older aged groups. In the very early stages of development studies have been made by Sabin (3) both in living preparations and histologically. According to her investigations a continuous differentiation of mesenchymal cells to angioblasts takes place in the first 2 days. These cells develop first in the membranes which are of interest to us. Angioblasts differentiate further during the course of the 3rd and 4th days of incubation. The angioblasts give rise to endothelium, blood plasma and blood islands. Only those structures are called blood islands which develop hemoglobin and become erythroblasts. The lumina of the blood vessels come into being through a process of cytolysis either in the centers of the cell masses or in the cytoplasm of single cells.

Our histological description begins at the 3rd day of incubation when the flow of blood in the vascular membrane has become established.

*Technique.*—Freshly opened eggs were fixed in formalin, Mueller formol or Zenker's solution. Blocks of paraffin or of gelatin were then made which were cut in series from the marginal veins to the embryo. Specimens of various ages were so prepared, cut either horizontally or transversely. They were stained with hematoxylin and Van Gieson's solution, resorcin-fuchsin and Sudan hemalum.

*Description of Embryos 4 Days Old.*—Radiating from the embryo there are ridges and folds which press into the yolk. In these folds the blood vessels run. The arteries lie in the folds nearer the yolk. They consist of single rows of flat or spindle cells, the circumference of the wall being the length of 10 to 12 cells. In the surrounding area there is a loose mesh network in which there are large nuclei. These cells do not belong to the arteries since they are also to be found without relation to them. Within the vessels there are free nucleated cells either round or spindle-shaped depending upon the aspect which they present. Laterally from

the arteries lie two veins. In comparison with the arteries they have a characteristic form. They are polygonal or flattened and adapt their shape to the space which is available, in this case, half surrounding an artery. Their wall is thin and consists of a single layer of cells. Arteries and veins are immediately juxtaposed so that at such places two layers of flat cells are to be found. There are no elastic fibers and no collagen. All vessels that belong to the yolk have the same form and are to be distinguished only by their size.

*Description of Embryos 10 Days Old. Vessels at a Distance from the Embryo.*—In the interval the dimensions of the arteries have increased to a five-fold extent. The wall turned towards the yolk consists of a single layer of cells. They contain a light staining protoplasm. The nucleus stains darker and contains many dark points arranged about a larger, more compact, area within. The relation of arteries and veins is less intimate; the veins run, at least in part, an isolated course.

*Vessels near the Embryo.*—Arteries have come to differ from veins through the distinct difference in thickness of their walls. On cross section the arteries appear to be round in contrast to the veins which are capable of taking on a variety of forms. The veins surround the arteries to the extent of a half to three-quarters of their circumference. The artery is embedded in the loose web-like tissue of the embryonic membranes. Their walls are about 5 cells thick. Their arrangement is lamellar. The nuclei are arranged in the long axis. The protoplasm appears more concentrated and is less in amount. The inner layer consists of somewhat taller endothelium. Between the cells delicate fibers stained bright red with Van Gieson's solution are to be found. There are no elastic fibers and no fat. The veins exhibit, surrounding the single row of cells of which they are formed, a layer of cells somewhat loosely meshed, the cells being large and composed of light staining protoplasm. Between the largest and the smallest arteries transitions occur ranging in dimension from arteries presenting two to three layers of cells to those of the largest size. The arrangement is always circular and never longitudinal. The smallest blood vessels, namely capillaries, often appear close to arteries, touching their outermost layers, but do not penetrate their walls.

*Description of Embryos 14 Days Old. Vessels at a Distance from the Embryo.*—The smallest arteries are built like capillaries and are to be distinguished from them only according to their size. They are of exactly the same form and structure as were those described in embryos of an earlier age. Nearer the embryo there are likewise to be found larger arteries of 2 to 5 layers.

*Vessels near the Embryo.*—A large artery of about 12 layers is to be found, the lumen being lined with a single layer of endothelium. Surrounding this is a longitudinal layer three cells thick. Beyond is a circular layer seven cells thick. Outside the vessel there lie connective tissue cells irregularly distributed and loose meshed, the cells containing small nuclei. The protoplasm of the cells in the arterial wall stains brown with hematoxylin and Van Gieson. These are young muscle cells. Distributed among them there are a few collagen fibers stained pale red. In the loose meshed tissue which is to be regarded as the adventitia there are

a few collagen fibers but there are no elastic fibers and there is no fat. The veins have a larger lumen than the arteries and are built of single layers of endothelium and about two layers of cells. The cell layers appear to be looser meshed than in arteries.

*Embryos 18 Days Old. Description of the Vessels Far Removed from the Embryo.*—Arteries built like capillaries are to be found as well as small arteries such as have already been described.

*Vessels near the Embryo.*—The arterial trunk described in embryos 14 days old is to be found also here. The wall has become thicker. The nuclei are well stained and their structure unchanged. There is no evidence of nuclear degeneration, decrease in the size of the nuclei nor indeed of any other change. There is no fat in the wall nor any elastic fibers.

*At the Site of Transition to the Embryo.*—The form of the artery is unchanged. There appear to be more collagen fibers. Sections stained with resorcin-fuchsin exhibit stoutly developed elastic fibers. In the outer third of the muscle layer there lies a broad band of elastic fibers passing in part into the adventitia. This zone consists of about five layers of short twisted elastic fibers which form a meshwork. There is a second layer of elastic fibers very fine in structure, which forms the internal elastic layer close under the single layer of endothelial cells. This structure also is not closely organized, but consists of single short slightly curved fibers which run circularly. Neither fat nor any other form of degenerative substance is to be found. This part of the artery exhibiting elastic fibers should no doubt be reckoned as being part of the embryo. This is the portion in which, as has been described in Part I, nerve fibers were found.

In the embryo itself in the early stages there are definite elastic rings in arteries of very much smaller dimensions than in the vascular membrane. The proof that elastic fibers occur in arteries within the embryo, whereas in those without there are none, is certain since in one and the same section both the embryo itself and the vascular membrane are frequently found.

## SUMMARY AND DISCUSSION

On the basis of the anatomical studies presented the following inferences or conclusions are drawn.

1. In the course of development there appear in the vascular membranes of chick embryos arterial vessels of all calibers, namely, capillaries, small arteries with 2 or 3 cell layers, and large arteries formed of endothelium, longitudinal and circular layers of muscle and adventitia.

2. In none of the stages are elastic fibers developed. Only in the most central portion of the umbilical artery, in that portion namely

which is to be regarded as belonging to the embryo, are elastic fibers discoverable.

3. The structure of capillaries is histologically the same at all stages. The small arteries of embryos 10 days old resemble histologically those of 18. At no stage of development are appearances of degeneration nor of fat to be found in arteries.

When the physiological results of our investigations are compared with the anatomical ones the following comments may be made. In respect to Paragraph 1 of the anatomical results we may remark that when we study the different forms of the wall of arterial vessels the most delicate vessels consisting of single cells exhibit the greatest irritability. Those which are built of 3 to 4 muscle layers are less irritable. Stouter vessels appearing for the first time at 10 days of incubation require stronger stimuli to bring about the same reaction. In respect to Paragraph 2 of the anatomical results we may make this comment. The absence of elastic fibers in all arteries of the embryonic membranes throughout the period of their development is important in defining a physiological property of the larger vessels.

The medium and larger vessels, beginning with the 4th day of incubation, contract differently from normal adult human arteries. In the contracted state they appear in cross section not as small replicas of larger circular structures, but take on a new form. During the course of contraction they become flat and appear band-like as would a garden hose when it is compressed by a weight. In examining a vessel so contracted one sees on rotating the vessel either a broad side or a narrow one. It is for this reason that such arteries appear alternately narrow as a line or broad as a band. It is not until the narrow artery is elevated with a hook that its uniform band-like nature becomes evident. The absence of elastic tissue, the presence of which in all probability is mainly responsible for the usual shape of arteries on cross section, permits one to see how the phenomenon which has been described may come about. Concerning Paragraph 3 of the anatomical conclusions we have this to say. According to the histological investigation a stage of degeneration is wanting in the blood vessels of the embryonic membrane in a sense in which one is accustomed to see such changes in other blood vessel systems during the course of life. On the day of hatching the constituent cells and fibers

of the arteries of all calibers are anatomically the same as in their early development. These vessels do not die as the result of aging: The nutrient fluid ceases to flow because of contraction of the umbilical vessels. The blood vessels die in complete possession of their physiological irritability and anatomical integrity. The unaltered irritability of blood vessels of the same caliber at all ages is consonant with their unaltered anatomical structure.

We wish to express our thanks to Miss Nelda Ricci for technical assistance.

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# STUDIES ON THE BLOOD VESSELS IN THE MEMBRANES OF CHICK EMBRYOS

## Part IV. Modification of Irritability of the Blood Vessels

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We studied the reactions of the blood vessels in the vascular membranes of chick embryos in Part II of this series. There we described changes which took place quantitatively and qualitatively in response to stimuli. It was our care in the earlier experiments to maintain the environment constant. In the present experiments we intended conversely to keep the stimulus constant but to change the environmental conditions. The question we have asked ourselves is, of course, whether with the same stimulus a difference in the irritability of the blood vessels can be brought about by altering the environment.

To obviate the danger of drying, due to the increased length of time required by these experiments, a substitute procedure was devised. We placed the eggs in Ringer's solution through which oxygen was bubbled. It was, of course, necessary first to be certain that this arrangement did not interfere with the viability of the embryos.

*Experiment 1.*—Eggs were opened on the second day of incubation, the albumen for the most part was drained off, and the yolk was laid in the Ringer's solution through which, as has been said, oxygen was continuously bubbled. At the beginning of the experiment the embryo with its membranes was 11 mm. in diameter, the blood vessels were not yet developed, and the embryo itself white in color. 24 hours after the beginning of the experiment the embryo and its membranes measured 14 mm., the heart was red and contracted 120 times per minute. In the blood vessels there was flow of red blood. At the end of 48 hours the embryo and its membranes measured 16 mm., the heart beat at the rate of 120, and there was

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vigorous flow in the vessels. On the 3rd day, the rhythm of contraction of the heart was irregular.

It is clear from these observations that the embryo including the circulatory apparatus grows normally for 2 days in this solution.

The demonstration of the viability of embryos and their membranes was sufficient to warrant the performance of experiments, the duration of which in no case exceeded 1 hour. We needed, of course, to be certain that oxygen in the concentration in which it was used exercised no effect on the irritability of the blood vessels of the embryonic membranes.

*Experiment 2.*—This was of a preliminary nature and was repeated in each case before proceeding to further tests. After opening the egg a branch of an artery was stimulated. Stasis occurred after the stimulus had been applied 3 times for 10 seconds. Then the preparation was irrigated with Ringer's solution through which oxygen was bubbled for 5 minutes. The vessel was again stimulated and stasis occurred again after 3 successive periods of stimulation each 10 seconds long.

We concluded that Ringer's solution through which oxygen was passed did not alter the irritability of the blood vessels.

We used carbon dioxide of known concentration in our experiments. It appears from the experiments of Hammett and Zoll (1) that saturated solutions of carbon dioxide bring about constriction of the vessels. Contraction takes place if the solution saturated with gas is injected under the membranes, as in the experiments of Hammett and Zoll, as well as when the surface is bathed with Ringer's solution which during this process is exposed to carbon dioxide in the usual fashion by bubbling the gas through the solution. These results we were able to confirm. We reduced, therefore, the concentration of carbon dioxide in Ringer's solution to a point at which it had no longer an effect upon the blood vessels.

*Experiment 3.*—Ringer's solution was placed in a glass burette having a capacity of 50 cc. Through this, air containing 5 per cent of carbon dioxide was bubbled so that 60 bubbles passed through in a minute. The egg after it had been opened was bathed with this solution. At the end of an hour the contractions of the heart remained unchanged, the rate being 128 per minute. No change took place in the diameter of the vessels, nor in the flow of blood through them.

We concluded that bathing the membranes with Ringer's solution containing carbon dioxide, the hydrogen ion concentration being between 7 and 6, would occasion no change in the rate of the heart or in the diameter of the vessels.

In view of the preliminary experiments which have just been described we adopted the following plan of operation.

A burette was provided with: (1) a tube through which oxygen was introduced, (2) a similar one for the delivery of carbon dioxide, and (3) a funnel through which salt solutions could be delivered. A tube led from the burette to a small glass cannula from which the solution designed to bathe the egg was permitted to drop. The perfusion stream was directed so that it fell not upon the embryo itself nor upon its membranes but on the surface at a distance from them, yet in such a way that the stream passed over the vascular layer. Direct impact of the fluid would have occasioned a mild stimulus the effect of which would have been characterized as we now know by dilatation of the vessels and a rapid rate of flow, the "state of fluxion."

Stimulation was supplied by means of a faradic current, the source being an Edison storage battery. To be certain that the current was constant, its voltage and amperage were continuously observed. The electrodes consisted of two copper wires a millimeter in diameter, placed a millimeter apart. With the exception of their poles they were carefully insulated. Observations were made with a binocular binobjective Zeiss microscope.

We measured the duration from the beginning of stimulation to the time of occurrence of stasis. To recognize this stage presented no difficulty because of the magnitude of the effect. Nor did this observation lead to error for the effect did not disappear and itself, therefore, served to control the observation. The experiments were all undertaken in a constant temperature room. Since the solutions and the gases were also kept here, the possibility of error due to alterations in temperature was excluded.

These arrangements permitted observations in which the vessels were stimulated alternately in milieux containing carbon dioxide and in others free of this gas.

After opening the egg: (1) Stimulation with a faradic current for 20 seconds was undertaken. The result was *stasis* of the blood stream. (2) When irrigation with Ringer's solution, *free* of carbon dioxide had been carried on for 2 minutes, stimulation with the faradic current was again applied for 20 seconds. *Stasis* again occurred. (3) When irrigation with Ringer's solution containing *carbon dioxide* had gone on for 3 minutes followed by stimulation with the faradic current lasting

150 seconds, *no stasis* appeared. (4) Next we irrigated the membrane with Ringer's solution which contained *oxygen* for 5 minutes and stimulated the vessels for 30 seconds. *Stasis* again appeared. (5) We irrigated again for 3 minutes with Ringer's solution containing carbon dioxide. This was followed by stimulation with a faradic current for 200 seconds, but *no stasis* occurred. Finally, we irrigated the preparation with Ringer's solution containing oxygen for 5 minutes and now stimulation with faradic current for 30 seconds only, was followed by the occurrence of *stasis*. Forty experiments like this were performed.

## DISCUSSION AND SUMMARY

When faradic stimulation was undertaken of vessels irrigated with Ringer's solution, which alternately contained and was free from carbon dioxide, it was observed that the reaction was far less when the solution contained carbon dioxide. A reversal of the effect could be obtained many times. It appears, therefore, that when Ringer's solution contained carbon dioxide in the concentration described, the irritability of the vessels to electrical stimuli decreased, although carbon dioxide by itself and in the absence of the application of the stimuli, appeared to be void of effect upon the vessels. The rare, divergent results were traced to technical errors.

We attempted to discover whether the observed decrease in irritability of the vessels might not be due to the absence of oxygen. For this purpose we irrigated the vessels with Ringer's solution alternately containing nitrogen and oxygen. When nitrogen caused any change this was due to an influence on the rate of the heart and not on the irritability or reactivity of the arteries. In whatever way we tried we were unable to bring about a change in reactivity of the arteries by creating a condition of oxygen lack independently of a change in the rate of the heart beat.

We attempted to study also the effect of other acids beside carbon dioxide on the changed reactivity of the arteries. Irrigation with various concentrations of lactic acid was without result. We also employed solutions buffered with potassium and sodium phosphate. When the irrigation was undertaken with these solutions having a pH range varying from 7.7 to 5.9 we observed neither a direct action nor

one which modified the preparation in such a way as to change its susceptibility to faradic stimulation.

Important investigations have been published recently by Atzler and Lehmann (2) on the direct influence of the hydrogen ion concentration on the behavior of blood vessels. Hammett and Zoll believed that, as the result of their experiments in which they attempted to bring about stimulation with solutions of concentrated carbon dioxide, they were able to exclude the possibility of action due to acid alone and therefore ascribed to carbon dioxide a specific effect. In our own experiments the method of irrigation does not permit an inference whether, or how far, an acid effect plays a rôle in the carbon dioxide experiments. For beside the question of hydrogen ion concentration and of buffering, the question of the penetration of substances from the surface to the contractile elements of the wall of the vessels requires to be considered. Carbon dioxide has an ability, beyond that of all other substances, to penetrate through tissues (3). It may be owing to this property that we could influence the reactivity of the blood vessels with it and it alone.

This possibility must be further investigated. In these experiments, however, it was our object to show only that it was possible to influence the irritability of blood vessels experimentally. The conclusion is justified by our experiments that carbon dioxide in small concentrations reduces the threshold of irritability for electrical stimuli of the blood vessels of the embryonic membrane.

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# ENHANCED PASSIVE IMMUNITY TO STREPTOCOCCUS INFECTION IN RABBITS

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We have been engaged for a number of years in studying the local intrapleural infection in rabbits produced by a strain of *Streptococcus haemolyticus* of increased and extreme virulence. These studies have shown that the rabbit dies uniformly of empyema on receiving a dilution of a 24 hour broth culture that contains not more than ten chains of the streptococcus. The resistance may be markedly increased, so that animals recover without lesions, by producing, with indifferent substances, cell accumulations in the wall of the pleural cavity provided that these accumulations be allowed to pass into the stage of mononuclear (clasmatocyte) cell infiltration (Gay, Clark and Linton).

Rabbits with a granulating pleura of this type are able to withstand many multiples of the lethal dose. It appears that this accumulation of clasmatocytes is effective not as a mechanical obstruction to infection but in virtue of the phagocytic value of its accumulated cells since inoculation in the opposite pleura, where no cell accumulation has taken place, also results in protection and can be shown to be due to a transpleural mobilization of the cells accumulated in the other cavity (Gay, Linton and Clark). It may further be shown that clasmatocyte mobilization in the peritoneum may be attracted through the diaphragm into an infected pleura and protect (Linton), and further that a transferred granulating omentum from one animal of the same species or even from an animal of another species (guinea pig into rabbit) may in a certain percentage of cases produce the same protection in a second animal. Throughout our experiments accumulations of polymorphonuclear cells have failed to produce the same protection. We feel justified in referring to this condition with the streptococcus as an "enhanced natural resistance" due to mobilization of mononuclear cells.

Unfortunately mononuclear mobilization although extremely effective in the streptococcus infection we have studied produces little or no result with some other microorganisms, for example with the pneumococcus and with *Pasteurella lepisepticus*. To consider more attentively the case of pneumococcus local infection it appears (Clark) that the mononuclear cells are by no means without function, for if the pneumococci have previously been treated with an immune serum (sensitized), mononuclear accumulations suffice, and alone suffice, to protect the animal. Such sensitized bacteria are uniformly fatal for normal rabbits or for rabbits with polymorphonuclear accumulations. These observations led us (Gay and Clark, 1929) to suggest an explanation for the failure of the majority of antibacterial serums to produce striking therapeutic results. This hypothesis is to the effect that such serums, directed against the bodies of bacteria rather than their toxins, are ineffective not because they lack appropriate or sufficient antagonistic properties (antibodies) but because the cells which are readily mobilized in the actively immune animal are lacking in the animal to which the serum has been passively transferred. The experimental results herewith reported are the first contribution which confirms this hypothesis.

A series of experiments with pneumococcus infection by various routes in rabbits seemed to indicate that a combination of homologous antiserum plus normal mononuclear cells is more effective in protecting a normal animal against infection than the serum alone. However, the antiserum obtained in rabbits is in itself so markedly protective that we do not regard our experiments as convincing as yet. On the other hand a return to our experiments with streptococcus seems to show in no uncertain way that cells increase markedly the protective effect of an homologous antiserum. As long ago as 1920 Gay and Stone showed that the serum of rabbits hyperimmunized against the streptococcus and containing various antibodies in considerable concentration was relatively ineffective in protecting rabbits against intrapleural infections with the same organism even when injected simultaneously with the infecting organism. With a dosage of 1 to 2 cc. of serum only 5 out of 20 animals (25 per cent) recovered. It should be repeated that the control animals always died uniformly from much smaller doses of organisms and in all attempted therapeutic

experiments of this sort at least 200 lethal doses are employed. In repeating these experiments recently we have obtained similar results. The intrapleural administration of from 0.2 to 2 cc. of rabbit antistreptococcus serum failed to protect 3 animals when given simultaneously and protected only 2 out of 10 animals when given 2 hours before the infecting dose. When the serum was administered intravenously from 1 to 24 hours before the streptococcus and in a dose of from 1 to 5 cc. only 1 animal out of 11 (9 per cent) survived.

From previous experiments (Gay and Clark, 1925) we find that although the serum in a single dose or several doses, beginning at the time of infection, is ineffective, protection may be regularly effected by giving antiserum 24 hours before the infection and again simultaneously with it. Under these conditions the pleural cavity becomes sterile within a few hours. On further analysis it was found that normal rabbit serum would also eventually result in sterilization although at a slower rate. We believe that we were able to show conclusively that this protective effect was associated with stimulation produced by the first dose, and mobilization produced by the second dose, of clasmatocytes in these protected animals. We did not at that time, however, point out the relation of antibodies to the more rapid result effected by the immune serum over the normal serum.

It remains to show that the presence of antibodies increases the considerably effective protection assured by clasmatocytes alone. This we have done in the following manner.

A. A series of 12 rabbits in several separate experiments were inoculated in the pleural cavity with streptococci corresponding in numbers to from 20 to 10,000 M.L.D. but previously exposed to sensitization by rabbit antistreptococcus serum in a dosage of from 0.2 to 0.5 of antiserum to 2 cc. of a 24 hour culture. These animals all died. In other words sensitization of the streptococcus by an immune serum does not protect the animal.

B. As we have already repeated, animals with pleural cavities prepared by injection of an aleuronat starch mixture 3 days previously are usually protected against direct inoculation of at least 200 M.L.D. of the streptococcus. When the infecting dose is given in the other cavity, unaffected directly by previous inoculation, protection may be evident although the results are uncertain and not effective against so

many lethal doses as in the originally prepared cavity. In this series 7 animals were prepared in the right cavity with aleuronat and starch and 3 days later injected in the left cavity with from 300 to 10,000 M.L.D. Of these animals 4 died and 3 survived (43 per cent). Of the survivors 2 received 300 and the other 1000 M.L.D.

C. Eight animals were prepared in the right cavity as in Series "B" and injected in the left cavity with 500 to 10,000 M.L.D. of sensitized culture. Of these animals 5 survived and 3 died (62.5 per cent). Those that died had received only 1000 M.L.D., whereas 3 of the survivors had received 10,000 M.L.D.

There seems no reason to question from these experiments that a combination of clasmatocytes (in themselves distinctly protective) and of antiserum (only slightly protective) is superior to either factor alone.

It is evident, however, that experiments of this type with sensitized bacteria do not correspond to conditions of natural infection with normal virulent organisms in spite of the fact that the sensitized cultures seem as fatal as untreated cultures.

We have previously shown (Gay and Clark, 1926) that the mononuclear exudate of an animal that has received aleuronat and starch 3 days previously and is itself protected has in the test tube no greater destructive property than the exudate (polymorphonuclear) of an animal prepared the day before and which is unprotected. In both instances the whole exudate fails to destroy streptococci but the supernatant fluid if immediately removed does have definite bactericidal properties. We further found that the mononuclear exudate of a nonspecifically protected animal has no power of transferring this protection to another animal. As much as 21 cc., in divided doses, beginning simultaneously with the infection, have been given to a rabbit without result. When, however, we employ the exudate of an actively immunized rabbit we find that the majority of animals treated with even small doses ranging from 0.2 to 4 cc. are protected in a majority of instances; 15 out of 20 rabbits (75 per cent) treated in this way with immune exudate survived. The exudate may be given either 2 hours before or simultaneously with the infecting dose and apparently with equally good effect.

Supernatant fluid of such an immune exudate alone, without the

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cells of the exudate, rarely protects. There were 2 survivors out of 15 animals (13⅓ per cent). It should be noted that in both of these experimental groups above cited protection seemed equally good when a 24 hour immune exudate was used as when a 3 day immune exudate (mononuclear) was employed. This seems a distinct difference from the contrast in protection in normal animals when these two grades of irritation have been produced in the pleura.

Further analysis shows that the cells alone of either mononuclear or polymorphonuclear exudate irrespective of whether they are from immune or normal animals do not protect and apparently the high degree of protection afforded by the whole immune exudate is due to the presence of antibodies in the supernatant fluid as well as cells. Such antibodies are always present though in slightly weaker titer than in the antiserum. It is evident that both cells and antibodies are necessary for if we add to the sediment of 1 cc. of either normal or immune exudate which may in turn be either polymorphonuclear or monuclear, an antiserum or an immune supernatant fluid the majority of animals are protected, 8 out of 9 or 89 per cent. Normal supernatant fluid plus cell sediment does not protect.

For some reason when *whole* exudate either immune or normal was added to antiserum the protection was less marked, that is only 2 out of 9 animals survived (22 per cent). We are not prepared to explain this discrepancy except to say that the total amount injected in these animals was usually larger than when the sediment of cells was employed and that failure may have been due to the volume or to excess of antibodies.

The experiments so far seem to show that a highly effective mononuclear cell mobilization is still further increased in protective efficiency by the presence of an antiserum which in itself is relatively ineffective, or conversely that the action of antistreptococcus serum in the experimental pleurisy in rabbits is definitely intensified in its effect if suitable cell accumulations are present at the seat of infection. Having established this main point it remains to seek a more effective adaptation to natural conditions of infection, particularly in the line of curative serum therapy. We can only suggest certain lines of investigation on which we are at present embarked.

If a normal exudate from the rabbit is given in the pleura 18 hours

previously and an antiserum given intravenously at the time of infection the animals survive (only three have been tested in this fashion). All the controls with the pleural treatment or with pleural treatment plus normal serum intravenously at the time of infection, died.

TABLE I

*Schematic Comparison of Degree of Protection against Streptococcus Pleurisy in the Rabbit Afforded by Different Methods*

| Preparation of animals | Dosage strept. | No. tested | Mortality | Degree of protection |
|---|---|---|---|---|
| Normal | 1 M.L.D. (10 chains)+ | 66+ | 100% | 0 |
| Normal with 24° polymorphonuclear exudate | 200+ | 8 | 100% | 0 |
| Normal | 20–10,000 *sensitized* | 12 | 100% | 0 |
| Normal with transferred normal exudate | 200+ | 8 | 100% | 0 |
| Antiserum locally | 1000 + M.L.D. | 33 | 77% | ± |
| Immune supernatant locally | 1000 + M.L.D. | 15 | 87% | ± |
| Antiserum intravenously | 1000 + M.L.D. | 11 | 91% | ± |
| Prepared 3 day (clasmatocyte), tested transpleurally | 300–10,000 M.L.D. | 12 | 50% | + |
| Prepared 3 day transpleural | 500–10,000 M.L.D. *sensitized* | 8 | 38% | + |
| Local normal serum—24°—normal serum | 200 + M.L.D. | 7 | 29% | ++ |
| Local antiserum—24°—antiserum | 200 + M.L.D. | 15 | 26% | ++ |
| Local immune exudate | 1000 + M.L.D. | 20 | 25% | ++ |
| Prepared 3 days, same side | 200 + M.L.D. | 14 | 14% | +++ |
| Local cells (normal or immune) + antiserum | 1000 + M.L.D. | 9 | 11% | +++ |
| (Local normal exudate + antiserum intravenously | 1000 + M.L.D. | 3 | 0 | ++++?) |

It is as yet uncertain whether the cells of normal or immune exudate are themselves active in increasing the protection by antiserum or whether they simply stimulate the local tissue to a rapid clasmatocyte response. The latter possibility is suggested by the fact that a polymorphonuclear exudate is as effective as a clasmatocyte exudate and

yet the donor of the early exudate is unprotected. We have not yet fully determined whether dried cells are as effective as fresh cells or whether local irritation with some other protein such as aleuronat or milk may do as well as cells. All attempts hitherto to produce a general stimulation of leucocytes by intravenous injection of vaccines, both specific and non-specific, or by indifferent proteins have failed to increase the effect of antiserum on the local infection.

In short, all that we feel justified in saying at the present time is that there appears from these experiments in local streptococcus infections in the rabbit definite evidence of the possibility of an enhanced passive immunity as previously defined, produced by a combined action of an antibacterial serum, which is in itself relatively ineffective, and normal cellular exudate locally, which in itself has no effect.

Table I is designed to give a schematic indication of the various methods of preventing experimental pleurisy which we have investigated.

## SUMMARY AND CONCLUSIONS

The experimental work herein reported tends to justify our hypothesis recently expressed, that the common failure of antibacterial serums to combat active infections when passively transferred to a normal animal, is due not so much to a lack of suitable or sufficient antibodies as to absence of cell preparation or mobilization in the recipient. In the case of experimental streptococcus empyema in the rabbit the course of the ordinarily fatal infection is in no wise affected by the transfer of the pleural fluid containing large numbers of mononuclear cells derived from an animal that is itself protected as a result of a non-specific irritation. The serum of a rabbit highly immunized against the streptococcus and containing antibodies for it, produces relatively slight effect in prevention or cure.

In contrast to this the pleural exudate, either acute (polymorphonuclear) or subacute (mononuclear), produced in an actively immunized animal does protect passively to a considerable degree. In a similar fashion normal exudate cells of either type in combination with the relatively ineffective antiserum give a high degree of protection.

It remains for further analysis to determine whether this form of passive immunity by antiserum enhanced by the addition of cells

depends on the vital properties of the cells transferred or on their stimulation to cell mobilization in the recipient. And furthermore the extent to which this enhanced passive immunity may be effective in cure, and whether the cure is applicable to local or to both local and generalized infection remains to be seen.

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# THE BLOOD CHEMISTRY OF AN ACUTE TRYPANOSOME INFECTION

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The recognition of the fact that the blood chemistry is altered has given a new direction to the study of experimental trypanosome infections.

Most of this work has dealt with the effect of the disease on the blood sugar, owing in part to the relative simplicity with which this factor may be studied, and in part to the claim of Schern, one of the first students of these changes, that the trypanosomiases are diseases in which the host's sugar is utilized by the parasites in a manner entirely analogous with that of bacteria growing in a test tube. The literature dealing with this point has been reviewed by Regendanz (1). Schern (2), von Fenyvessy (3), and Scheff (4), have published figures which show that the blood sugar concentration falls as the number of parasites increases, while other workers, amongst whom Regendanz (1) has published the most complete data, believe that the hypoglycaemia is only a terminal phenomenon. It has become evident that the true state of affairs in these infections is more complicated than Schern supposed, and in the field of carbohydrate metabolism difficulties of interpretation have increased rather than diminished as more investigators have reported their results.

Other blood changes have been much less thoroughly explored. Scheff (2) has published a report dealing with various aspects of the problem, beside the carbohydrate metabolism. He emphasizes the amount of oxygen consumed by the trypanosomes *in vivo*, a phenomenon which gives rise to an oxygen lack in the host, and leads eventually to death by "inner asphyxia." The upset in oxygen metabolism, the exhaustion of the free and reserve carbohydrates, and the probable presence of a toxin, determine the death of the host, in Scheff's opinion.

Scheff's data on the oxygen content of the blood could not be confirmed by Kligler, Geiger, and Comaroff (5). They found but little difference in this respect between normal and infected rats. Nor were the experiments which they devised to show a toxin successful. They incline to the view that the rapid multiplication of the parasites leads to the production of lactic acid in quantities which cannot be

successfully cared for by the host. The oxidative metabolism is thus upset, the alkali reserve depleted, and death ensues.

Discordant results have also been reported in regard to the liver glycogen. Scheff's data point to an exhaustion of this substance as the infection progresses, while Regendanz believes that the liver glycogen is always present in sufficient amount to keep the blood sugar at a normal level, and that the terminal lowering of the glucose is due to the action of a trypanosome toxin on the glycogen-glucose mechanism.

The strain of *T. equiperdum* was obtained from the Hygienic Laboratory in Washington. In rats it produces an acute septicaemic infection which terminates fatally in from 72 to 96 hours, after an incubation period which varies with the infecting dose. The majority of animals will not survive a concentration of trypanosomes much greater than one million per cubic millimeter of blood, although occasionally a concentration of nearly two million is observed. The rat does not show any resistance to the invader, so far as can be discovered. On the other hand, individual animals show marked variations in the number of parasites which they can tolerate before death ensues. Furthermore, the speed of reproduction as shown by the length of time in which the number of parasites doubles, shows considerable variation from rat to rat. Whether these are the results of individual resistance cannot be stated. It is clear, however, from the total counts, that the infection is by no means such a clear-cut, mathematically reproducible one as other workers have described for other strains.

Our present report deals with the foregoing matters and with certain other factors which have not yet been emphasized in trypanosome infections. These are points of attack by the parasites on the host's metabolism, the understanding of which is necessary for a complete picture of the disease. We have been interested in this infection also because of the information it may yield for the study of acute septicaemias in general. Infection with our strain of *Trypanosoma equiperdum* in the rat presents a relatively uncomplicated septicaemia: the infectious agents are confined nearly exclusively to the blood stream, while other tissues are involved to only a slight and irregular degree, and then only when the disease has almost run its course. Antibody production and leucocytic reactions are absent or evident to only a very slight degree; and the reticulo-endothelial system, the fundamental importance of which is well recognized in a large number of infections, is not involved (Dwijkoff (6)).

### *Methods*

The $CO_2$ combining capacity, non-protein nitrogen, uric acid, and chloride determinations were made by the usual laboratory methods.* Micromethods were used for the other determinations, as follows: Liver glycogen—method of Slosse (7); blood sugar—method of Folin (8); lipoid phosphorus—method of Krasnow and Rosen (9); cholesterol—method of Ling (10). Blood was obtained by heart puncture under light anaesthesia with iso-amyl-ethyl barbituric acid (amytal), approximately 7 mg. being given per 100 gm. of rat. In a few instances where the animals were *in extremis* anaesthesia was not used.

The data on the animals are arranged in the tables according to increasing numbers of trypanosomes per cubic millimeter of blood. It must be emphasized, however, that this arrangement shows only roughly the increasing severity of the disease. With this strain of trypanosomes, and with the stock of rats used, the lethal concentration varies greatly from one animal to another. For example, Rat 9, in Table II, with 925,000 organisms per cubic millimeter was moribund when bled, while Rat 16 in Table I, showing twice as many trypanosomes, still had several hours of life, to judge from its general appearance, breathing, activity, etc. This example is the most extreme one that occurs in our data, but it serves well to illustrate that the arrangement of figures shows a relative rather than an absolute severity of infection.

### *Carbon Dioxide Combining Capacity*

In Table I are presented the data for the $CO_2$ combining capacities of 14 infected and 2 normal rats. Beginning with the fourth animal in the list (137,000 trypanosomes per cubic millimeter) all the infected rats show a well defined lowering of the $CO_2$ capacity. An acidosis is thus present from an early stage of the disease, varying in intensity from moderate to severe. The acidosis does not vary with the progress of the infection, as is shown by the figures for Rats 15 and 16, but drops while the number of trypanosomes is still small to varying levels below normal, and remains subnormal throughout.

### *Non-Protein Nitrogen, Uric Acid, Chlorides*

The data on these constituents are presented in Table II, which gives the figures for 4 normal and 10 infected animals. Up to and including Rat 7 the figures fall within the normal ranges, but beginning with

* I wish to thank Mr. S. Graff, of the Department of Biochemistry, for these determinations, and Mr. R. Rebold, of the same department, for the sugar determinations on the hydrolized glycogen.

a higher concentration of trypanosomes (760,000 per cubic millimeter) there is a definite rise in the non-protein nitrogen and in the uric acid to figures which indicate pathological changes in the kidneys. These changes appear as preterminal rises, occurring at a time when 4 to 6 hours represents the estimated length of life.

Histologically the kidneys are normal until a late period in the disease. The changes which they show are those of an early nephrosis. The blood vessels are greatly swollen and in some areas hemor-

TABLE I

*Carbon Dioxide Capacity of Serum in Rats Infected with Trypanosoma equiperdum*

| Rat No. | Trypanosomes per cu. mm. | $CO_2$ capacity. Vol. per cent |
|---|---|---|
| 1 | Normal | 59.5 |
| 2 | Normal | 62.0 |
| 3 | 75,000 | 65.0 |
| 4 | 137,500 | 37.0 |
| 5 | 200,000 | 13.6 |
| 6 | 225,000 | 45.0 |
| 7 | 275,000 | 35.0 |
| 8 | 525,000 | 25.0 |
| 9 | 820,000 | 47.8 |
| 10 | 825,000 | 36.0 |
| 11 | 830,000 | 31.9 |
| 12 | 850,000 | 20.2 |
| 13 | 1,000,000 | 31.5 |
| 14 | 1,125,000 | 14.5 |
| 15 | 1,175,000 | 46.0 |
| 16 | 1,850,000 | 40.4 |

rhages have occurred; the glomeruli are enlarged and fill the capsules; sections stained with Giemsa show them to be swarming with trypanosomes. They seem, however, to be intact, and undamaged, and the same is true of the collecting tubules. The secreting tubules, however, have undergone marked changes; they are swollen, and in some places the cells are broken down; fat has appeared in them, in the form of droplets, in large amounts, while it is absent in the glomeruli and in the collecting tubules. In some cases albumin is present in the urine.

The chlorides do not show any significant variations. In the three

TABLE II

*Non-Protein Nitrogen, Uric Acid, and Chlorides in the Blood of Rats Infected with Trypanosoma equiperdum*

| Rat No. | Trypanosomes per cu. mm. | Non-protein nitrogen. Mg. per 100 cc. | Uric acid. Mg. per 100 cc. | Chlorides. Mg. per 100 cc. | |
|---|---|---|---|---|---|
| | | | | Blood | Serum |
| 1 | Normal | 39.6 | 2.8 | 482 | 590 |
| 2 | Normal | 33.0 | 3.0 | 461 | 592 |
| 3 | Normal | 29.0 | 1.9 | 453 | — |
| 4 | Normal | 32.0 | 2.6 | 468 | — |
| 5 | 475,000 | 37.0 | 2.9 | 470 | — |
| 6 | 525,000 | 36.0 | 3.1 | 432 | 573 |
| 7 | 600,000 | 36.4 | 2.7 | 465 | 575 |
| 8 | 760,000 | 42.0 | 4.8 | 472 | — |
| 9 | 925,000 | 138.0 | 11.3 | 381 | — |
| 10 | 1,050,000 | 73.0 | 7.8 | 420 | 566 |
| 11 | 1,100,000 | 47.0 | 5.6 | 459 | 572 |
| 12 | 1,275,000 | 57.0 | 5.6 | 453 | — |
| 13 | 1,450,000 | 56.0 | 6.1 | 470 | — |
| 14 | 1,800,000 | 40.0 | 3.9 | — | — |

TABLE III

*Lipoid Phosphorus and Lecithin in Whole Blood of Rats Infected with Trypanosoma equiperdum*

| Rat No. | Before infection | | After infection | | Percentage increase | Trypanosomes per cu. mm. |
|---|---|---|---|---|---|---|
| | Lipoid P. Mg. per 100 cc. | Lecithin. Mg. per 100 cc. | Lipoid P. Mg. per 100 cc. | Lecithin. Mg. per 100 cc. | | |
| 1 | 8.05 | 201 | 10.35 | 258 | 28 | 25,000 |
| 2 | 7.05 | 176 | 9.22 | 230 | 30 | 292,000 |
| 3 | 9.25 | 231 | 10.00 | 250 | 8 | 300,000 |
| 4 | 8.9 | 222 | 10.85 | 271 | 21 | 575,000 |
| 5 | 8.1 | 202 | 11.75 | 293 | 42 | 575,000 |
| 6 | 8.9 | 222 | 10.8 | 270 | 21 | 600,000 |
| 7 | 9.6 | 240 | 12.5 | 312 | 30 | 625,000 |
| 8 | 7.8 | 195 | 10.55 | 253 | 35 | 740,000 |
| 9 | 8.8 | 220 | 13.3 | 332 | 51 | 850,000 |
| 10 | 9.05 | 226 | 11.6 | 290 | 28 | 1,200,000 |
| 11 | 8.8 | 220 | 17.5 | 437 | 98 | 1,800,000 |

instances in which they differ at all markedly (Rats 6, 9, and 10) they are lower than the normal range.

### *Lipoid Phosphorus and Lecithin*

Table III contains the data for lipoid phosphorus, and the calculated lecithin content in eleven animals before and after infection. Without exception the animals show increases in these constituents. The in-

TABLE IV

*Cholesterol in Whole Blood in Rats Infected with Trypanosoma equiperdum*

| Rat No. | Cholesterol before infection. Mg. per 100 cc. | Cholesterol after infection. Mg. per 100 cc. | Trypanosomes per cu. mm. |
|---|---|---|---|
| 1 | 76 | | |
| 2 | 81 | | |
| 3 | 68 | | |
| 4 | 71 | | |
| 5 | 87 | | |
| 6 | 80 | | |
| 7 | 86 | | |
| 8 | 82 | | |
| 9 | 80 | 74 | 717,000 |
| 10 | 74 | 76 | 750,000 |
| 11 | 67 | 67 | 820,000 |
| 12 | 76 | 85 | 830,000 |
| 13 | 73 | 69 | 850,000 |
| 14 | 76 | 78 | 900,000 |
| 15 | 74 | 84 | 1,250,000 |
| 16 | 76 | 83 | 1,275,000 |
| 17 | 66 | 69 | 1,850,000 |

crease begins very early in the course of the infection, when the parasites have been in the blood but a few hours (Rat 1), and has become nearly twice normal when the disease ends. With two exceptions (Rats 3 and 11) the increase varies between approximately 20 per cent and 50 per cent of the figure obtained from the normal animal.

### *Cholesterol*

Cholesterol determinations are given in Table IV. The figures show that no changes in this blood constituent occur as the result of

the trypanosome infection. The amounts of cholesterol during the severe stages of the infection are all within the normal range, and vary but a few milligrams from the determinations before infection.

TABLE V

*Liver Glycogen, as Glucose, and Blood Sugar, in Rats Infected with Trypanosoma equiperdum*

| Rat No. | Trypanosomes per cu. mm. | Grams of liver taken | Glucose. Mg. per gram of liver |
|---|---|---|---|
| 1 | Normal | 1.162 | 20.1 |
| 2 | Normal | 1.117 | 16.3 |
| 3 | 87,500 | 1.043 | 0 |
| 4 | 100,000 | 1.269 | 0 |
| 5 | 112,500 | 2.085 | 3.7 |
| 6 | 137,000 | 1.064 | 6.0 |
| 7 | 225,000 | 1.108 | 3.4 |
| 8 | 300,000 | 1.198 | 7.0 |
| 9 | 500,000 | 0.688 | 0 |
| 10 | 660,000 | 2.426 | 0 |
| 11 | 825,000 | 1.172 | 0 |
| 12 | 1,000,000 | 1.081 | 0 |
| 13 | 1,075,000 | 2.098 | 0 |

*Blood Sugar*

| Rat No. | Trypanosomes per cu. mm. | Blood sugar. Mg. per 100 cc. |
|---|---|---|
| 1 | 112,500 | 67 |
| 2 | 292,000 | 112 |
| 3 | 300,000 | 141 |
| 4 | 575,000 | 79 |
| 5 | 660,000 | 88 |
| 6 | 740,000 | 111 |
| 7 | 1,200,000 | 75 |

### *Liver Glycogen and Blood Sugar*

Determinations of liver glycogen and blood sugar are given in Table V. The two rats in the earliest stages of the infection were without glycogen demonstrable by the method used. The other rats which were positive had low glycogen values, compared with the controls. The last five animals, with counts of 500,000 trypanosomes per cubic

millimeter and over, did not show any glycogen, even when, as in two of the cases, over 2 gm. of liver were taken. Histologically, the livers of these animals show an enormous accumulation of fat.

## DISCUSSION AND SUMMARY

Our results show that animals infected with *T. equiperdum* suffer from an acidosis that is present continuously from an early stage in the disease, and that this condition is complicated subterminally by the breaking down of the kidney structure. It is difficult to estimate how much influence the renal condition may have on the animal's death, but it is probably an important contributory cause.

The increase in lecithin may be of significance in relation to the disappearance of the liver glycogen and the maintenance of a normal blood sugar level. It is known that fat circulates in the blood as a lecithin-like compound (Bloor), and the production of sugar from fat, and of dextrose from glycerol (12), in the animal body, appears to be possible. For trypanosome infections, however, such statements can only be considered as suggestive possibilities, particularly in view of the fact that a hypoglycaemia develops while large amounts of fat are still present in the liver.

The absence of any change in the blood cholesterol in *T. equiperdum* infections is in contrast with the changes found for this constituent in various diseases such as typhoid fever, scarlet fever, and erysipelas; and in chronic infections such as malaria and syphilis. Our results also differ from those of Dubin (13) on one dog infected with this same organism. In this case, the infection was presumably a chronic one, and the dog had a cholesterol and lecithin blood content lower than normal. Our results for normal rats, using Ling's technique, are about 10 per cent lower than those reported by Randles and Knusson (14), who used Bloor's method.

## SUMMARY

The $CO_2$ capacity of the serum is markedly lowered early in infection with *Trypanosoma equiperdum*.

The non-protein nitrogen and uric acid constituents of the blood are increased in the terminal stages. The kidneys also show terminal degenerative changes.

The cholesterol remains unchanged throughout.

Lecithin is markedly increased, most of the observations showing a 20 per cent to 50 per cent rise in this substance.

Liver glycogen is lower than normal in the early stages and could not be demonstrated in the later stages of the infection.

The blood sugar remains normal until a very late period in the disease.

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# THE SURVIVAL OF YELLOW FEVER VIRUS IN CULTURES

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During the period from January to June, 1929, blood and tissues from many *rhesus* monkeys, experimentally infected with yellow fever, were planted in a variety of artificial media. Cultures were made, also, from several samples of human blood from patients with yellow fever; but these were prepared under less favorable circumstances than the cultures of monkey material, since there was usually a lapse of several days between the withdrawal of the sample and its arrival at the laboratory. The great majority of the cultures remained bacteriologically sterile, as determined by the absence of visible growth and our failure to find organisms by dark field examination and in stained smears.

## *Injection of Cultures into rhesus Monkeys*

Cultures were injected into *Macacus rhesus*, and some of the inoculated animal subsequently had febrile temperatures; in a few instances it was possible to prove that yellow fever had really been contracted. In Table I a brief summary is given of nine experiments in which the proof was complete.

The longest period intervening between inoculation of culture medium and injection therewith of a test monkey, with resulting yellow fever, was 12 days. In this instance, McNeal-Marchoux medium had been employed, but the most consistently satisfactory results were obtained with a special egg medium. This was a modification of McCoy's egg-yolk medium (1) for the cultivation of *B. tularense*. Its composition and mode of preparation were as follows:

| | |
|---|---|
| Egg yolk | 110 gm. (approximately) |
| Rabbit blood (defibrinated) | 40 cc. |
| Distilled water | 50 cc. |

The ingredients were thoroughly mixed, coagulated in a slanted position in the Arnold sterilizer, and sterilized fractionally at a temperature of 75°C. In later

* This paper was prepared by Dr. N. C. Davis from the laboratory notes of Dr. Paul A. Lewis, who died of yellow fever in Bahia, Brazil, June 30, 1929.

TABLE I

*Yellow Fever Infections in Monkeys Following Injection of Cultures Inoculated with Virus*

| Material cultured | Source of inoculum | Medium | Length of time in culture (days) | Quantity infectious blood injected (cc.) | Monkey inoculated | Strain of virus | Outcome | Criteria of infection |
|---|---|---|---|---|---|---|---|---|
| Liver | *Rhesus* H | Egg-Serum | 2 | | *Rhesus* L | Asibi | Killed when moribund | Typical lesions in liver |
| Blood | *Rhesus* A | Egg-Serum | 4 | 0.25 | *Rhesus* B | Asibi | Killed on 2nd day of fever | Typical lesions in liver |
| Blood | *Rhesus* A | Egg-Serum | 4 + 4* | ? | *Rhesus* C | Asibi | Killed on 4th day of fever | Typical lesions in liver |
| Blood | *Rhesus* A | Egg-Serum | 8 | 0.5± | *Rhesus* D | Asibi | Killed when moribund | Typical lesions in liver |
| Blood | *Rhesus* A | Marchoux | 12 | 0.5± | *Rhesus* E | Asibi | Killed on 2nd day of fever | Positive transfers of blood. (Liver lesions not definite) |
| Blood | *Rhesus* B | Egg-Serum | 9 | 0.2 | *Rhesus* J | Asibi | Killed when moribund | Typical lesions in liver |
| Blood | *Rhesus* E | Egg-Serum | 4 | 0.000033‡ | *Rhesus* K | Asibi | Killed when moribund | Typical lesions in liver |
| Blood | *Rhesus* U | Noguchi† (semi-solid) | 7 | 0.1 | *Rhesus* Z | S.R. | Fever. Recovered | Serum protected against virus |
| Blood | *Rhesus* U | Noguchi (semi-solid) | 7 | 0.1 | *Rhesus* AA | S.R. | Fever. Recovered | Positive transfer of blood. Animal afterward immune |

* First condensation water from culture tubes injected into *Rhesus* B. Fluid replaced and injected into *Rhesus* C at end of second 4-day period.

† Culture also contained *Leptospirae*.

‡ Only condensation water and washings were injected; clot of original blood (diluted with normal blood) was not used.

experiments part of the egg yolk in this mixture was replaced by monkey liver and the medium was heated to 100°C. However, in the experiments in which the latter mixture was used, the results were negative.

A number of trials were made with the "MON" medium recommended by Kuczynski (2). It was impossible, however, to obtain the "normosal" called for in this formula, so a 1:5 dilution of sea-water was used instead. It is believed that none of the animals which were injected with cultures made on this medium contracted yellow fever. In one series the original quantity of blood was small (0.0001 cc.), but if the medium had been as favorable to the multiplication of the causative organism as its originator claimed, there should have been no trouble in securing an infection at the end of 4 days. In another series, the quantity of infectious blood in each tube was about 0.1 cc., but the incubation of the cultures lasted 7 days.

One series of experiments was made with Noguchi's semisolid *Leptospira* medium. Some of the inoculated tubes were left unsealed; the others were sealed with vaseline (partial anaerobiosis). None of the monkeys injected with these cultures developed yellow fever. In a second series, half of the tubes were inoculated with *Leptospira icteroides* (strain Palmeiras V) in addition to the infectious blood, since it was thought that the virus might maintain itself better in symbiosis with *Leptospirae*. At the end of 1 week mild infections were obtained by the injection of media both with and without *Leptospirae*; injections at the end of 2 weeks did not produce infection.

In a series of fifteen tubes of coagulated egg medium, the condensation fluid was supplemented by an egg-liver-brain extract with a trace of some substance for enrichment, either dextrose, lactose, maltose, sucrose, mannitol, glycerol, glycogen, peptone, or tryptic digest; and to this was added 0.001 cc. of infectious blood diluted to 0.05 cc. with normal monkey blood. No infections resulted from injections of this material at the end of 1 week.

The virus used in cultures represented three strains (Asibi, B.B., and S.R.) in routine use at the laboratory in Bahia (3). Except for the two cultures in semisolid medium with S.R. strain virus, all successful inoculations were with the Asibi strain. Cultures in Noguchi's medium were maintained at room temperature (22 to 25°C.); the other cultures were incubated at 35°C.

In addition to the experiments in which yellow fever was produced (see Table I), there were a number of others in which, without definite proof of infection, suggestive evidence was obtained.

The most interesting of these concerned *Rhesus* Q, which was injected with material from a culture on egg medium 18 days old. The animal had fever on the second and third days following injection; the maximum temperature was 105.2°F. 16 days after the first injection he was given an immunity test with fresh infectious blood and again had fever, this time for 6 days, with a maximum temperature of 104.6°F. Recovery followed.

*Injections of Di*

| Quantity of blood (cc.) | Experiment I. May 28, 1929 | | | Experiment II. June 7, 1929 | | |
|---|---|---|---|---|---|---|
| | Strain of virus | Animal inoculated | Outcome | Strain of virus | Animal inoculated | Outco |
| 3.0 | | | | | | |
| 1.0 | Asibi | *Rhesus* F | Killed on 2nd day of fever. Lesions indefinite | | | |
| 0.1 | Asibi | *Rhesus* G | Fever. Dead on 4th day. Typical yellow fever | | | |
| 0.01 | Asibi | *Rhesus* H | Fever. Killed when moribund on 4th day. Typical yellow fever | | | |
| 0.001 | Asibi | *Rhesus* I | Fever. Killed when moribund on 5th day. Typical yellow fever | Asibi | *Rhesus* P | Fever for 5 d covered |
| 0.0001 | | | | Asibi | *Rhesus* O | Fever. Died Typical ye |
| 0.00001 | | | | Asibi | *Rhesus* N | Fever. Kil moribund o Typical ye |
| 0.000001 | | | | Asibi | *Rhesus* M | No fever. D day. Prob fever |

*ctious Blood*

| Experiment III. June 11–14, 1929 | | | Experiment IV. June 20–24, 1929 | | |
|---|---|---|---|---|---|
| Strain of virus | Animal inoculated | Outcome | Strain of virus | Animal inoculated | Outcome |
| S.R. | *Rhesus* S | Fever. Killed when moribund on 4th day. Typical yellow fever | | | |
| S.R. | *Rhesus* U | Fever. Killed when moribund on 6th day. Typical yellow fever | S.R. | *Rhesus* BB | No fever. No reaction to immunity test |
| S.R. | *Rhesus* R | Fever. Killed when moribund on 5th day. Typical yellow fever | S.R. | *Rhesus* Y | Killed on 2nd day of fever. Typical yellow fever |
| S.R. | *Rhesus* T | Fever. Killed when moribund on 5th day. Typical yellow fever | S.R. | *Rhesus* X | Fever. Killed when moribund on 6th day of fever. Typical yellow fever |
| | | | S.R. | *Rhesus* W | No fever. No reaction to immunity test |
| | | | S.R. | *Rhesus* V | Fever. Killed when moribund on 7th day. Typical yellow fever |

Many animals that showed no temperature reaction to the original injection later proved partly or wholly refractory to test doses of virus; the assumption is that some immunity was called forth by the dead or attenuated virus present in the cultures.

*Rhesus* B was inoculated with the condensation water from two culture tubes at the end of 4 days' incubation, containing in all about 0.25 cc. of infectious blood. The fluid from these tubes was replaced with that from uninoculated medium, and incubation was continued for 4 days more. *M. rhesus* C was inoculated with the fluid from these same tubes at the end of the second 4-day period. Although *Rhesus* B and *Rhesus* C were sacrificed while still in the febrile stage, both had liver lesions typical of yellow fever. It was thought at first that there might have been reproduction of the virus in the culture medium, but the results of dilution experiments suggested that probably there was merely a survival from the original inoculum.

### *Injections of Infectious Blood Highly Diluted*

A summary of the dilution experiments is given in Table II. It will be noted that while Experiment III and Experiment IV each represents a single series of dilutions, in neither case were all injections made on the same day or with blood from the same monkey. Each of these experiments might be subdivided, making six in all, except that additional space would be needed for recording them and the results are sufficiently clear in the present scheme. Dilutions of infectious blood were made in every case with either citrated or defibrinated blood from normal monkeys. Injections were made intraperitoneally.

It is evident that both the Asibi and S.R. strains of virus were fatal in quantities of 0.00001 cc. of infectious blood. There is no reasonable doubt that the monkey which received 0.000001 cc. of blood containing Asibi strain virus also succumbed to yellow fever. The liver section showed a considerable number of postmortem changes, but there was undoubtedly a severe injury preceding death. Aragão (4) records positive results from the injection of a millionth part of an infected mosquito.

## DISCUSSION

It has been shown by Sawyer, Lloyd, and Kitchen (5) that citrated or clotted blood from animals infected with yellow fever retains

a certain amount of virulence after storage for at least 35 days and that glycerinated blood is capable of infecting animals after preservation for 60 days. Their specimens were kept at refrigerator temperature. The dosage which produced yellow fever was 1 cc.

From the results of the dilution experiments recorded above, it would seem certain that the infections obtained from the injection of cultures were due to the survival of virus from the original inoculum. The only surprising point is that virus survived in infective quantities for at least 12 days at a temperature of 35°C., when usually it dies out quite rapidly in citrated blood at room temperature.

It is believed that such of the infections reported by Kuczynski (2) as were actually yellow fever, were caused by virus which survived in the medium and not by the visible organisms under cultivation. Since extremely minute quantities of virus will produce infection, it is easy to account for the virulence of subcultures. This explanation does not preclude the possibility that the bacteria of Kuczynski, although probably not the cause of yellow fever, may have had some pathogenic properties of their own.

## SUMMARY AND CONCLUSIONS

1. The virus of yellow fever has been found to survive in artificial culture media for at least 12 days at a temperature of 35°C. No visible growth has been present and no reproduction of the virus has been demonstrated.

2. Infections have been obtained in *rhesus* monkeys with two strains of virus in quantities as small as 0.00001 cc. of infectious blood, and with one strain in an amount probably as minute as 0.000001 cc.

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# STUDIES ON BARTONELLA MURIS ANEMIA OF ALBINO RATS*

## I. Trypanosoma lewisi Infection in Normal Albino Rats Associated with Bartonella muris Anemia

## II. Latent Infection in Adult Normal Rats

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(Received for publication, April 24, 1930)

It has been observed (1, 2) that removal of the spleen in adult albino rats is followed by a severe anemia within a few days to 3 weeks. This anemia is characterized by a marked drop in the normal red cell count from 8 to 10 million cells per cubic millimeter to 1 million or less. There is a corresponding drop in the hemoglobin and the appearance in the red blood cells of inclusion bodies known as *Bartonella muris* bodies. These bodies appear as diplococci and rods on the surface of the cell. They occur in very large numbers (20 to 30 per cell) at the height of the anemia. Poikilocytes, anisocytosis, polychromasia and normoblasts are present in the circulating blood. Erythrocytosis by large circulating mononuclears is occasionally observed. Noguchi has succeeded in growing a bacillus for one generation from the blood of infected rats on a special *Leptospira* medium, to which blood has been added (3). The failure of other investigators to isolate these organisms has cast some doubt on the etiological rôle played by these bodies in the anemia (4). Nevertheless their persistent appearance coincidental with the anemia is striking.

All the rats used in our studies have been raised in our laboratory from original Wistar Institute stock. The anemia develops in the adult rats of this stock following splenectomy in all instances. About 20 per cent of the rats die within 8 to 10

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* Read before the American Association of Pathologists and Bacteriologists, April 17, 1930.

days from anemia. The anemia begins as early as the 3rd day and as late as the 16th day. It is accompanied by a severe hematuria. The disease is rapidly progressive, reaches its peak about the 8th to the 12th day and then diminishes. In those recovering from the disease it disappears after 3 to 5 weeks, the count returning to the normal level. Recurrences occasionally occur, but if the rat survives the height of the first attack it survives subsequent attacks. At times the onset is precipitous, the number of red blood cells per cubic millimeter dropping to 2 or 3 million within 24 hours after the onset. Males develop the anemia more rapidly than females. It has been observed that some strains of rats do not develop the anemia (5).

Autopsy reveals a profound anemia of the organs and marked icteric tint to the subcutaneous fat. There is generally free fluid in the serous cavities, the blood in the heart is unclotted and watery. The liver, kidneys and heart show marked fatty changes. The bone marrow is red. Microscopically there are focal areas of necrosis in the liver. The Kupfer cells are markedly distended with engulfed red blood cells, hemosiderin pigment and *Bartonella muris* bodies. In general the reticulo-endothelial system is severely damaged. The kidneys reveal an extensive nephrosis and in some instances embolic hemorrhagic glomerular nephritis. The glomeruli may show necrotic changes, the tubules show marked degeneration and their lumina are filled with albuminous material and iron containing pigment and occasional casts of red blood cells. There may be small areas of infarction in the kidneys without any surrounding inflammatory response. This is most striking in young rats with a fatal infection. In very anemic young rats with intact spleens, the pulp cells show extensive degenerative changes and there are areas of necrosis.

From the work of Lauda (1) and Mayer (2) the infectious nature of the anemia had been suspected, but the anemia could not be transmitted from the splenectomized rat to any adult normal animal. Ford and Eliot (4) were able to transmit this anemia through many passages in immature normal rats under 40 gm. in weight and immature normal rabbits by direct injection of whole blood of a splenectomized anemic rat. This would indicate a difference in the functional activity of the spleen in the young and adult animal. It would suggest that the adult normal rat is resistant to the anemia due to a permanent immunity from an infection with the "virus" of *Bartonella muris* anemia in early life. This immunity is broken down in the adult rat by the removal of the spleen and a second invasion with the virus occurs. It is possible, however, that the virus remains latent in the adult rat following an early infection and that the adult normal rat though immune is a "carrier."

An effort was made to determine experimentally (a) whether the adult normal rat is a "carrier" of the virus of the anemia; (b) whether injury to the spleen by a protozoan infection permits the occurrence of an anemia and if so, whether such an anemia is identical with the *Bartonella muris* anemia.

### *1. The Transmission of a Strain of the Bartonella muris from Splenectomized Adult Rats to Normal Young Rats and Rabbits*

The anemia of splenectomized rats may be transmitted to young rats under 40 gm. and to 3 week old rabbits. This observation made by Ford and Eliot has been confirmed by us.

The injection of 0.5 cc. of whole blood from a splenectomized rat intravenously into 3 week old rabbits is followed by a profound anemia within 10 days which persists for several days and within 3 weeks the blood count returns to normal. The anemia can then be transmitted to baby rats for many transfers.

*Sample Protocol.*—Rabbit 4 injected intravenously with 0.5 cc. of whole blood from a splenectomized rat at height of the anemia.

| | Blood count | Hbg. (Dare) | *Bart.* bodies |
|---|---|---|---|
| * 1/16—Before injection..... | 6,350,000 | 105% | |
| 1/20—After injection..... | 6,600,000 | 90% | |
| 1/22 " " ..... | 3,900,000 | 37% | Numerous rods and diplococci |
| 1/25 " " ..... | 1,800,000 | 10% | " |
| 1/28 " " ..... | 1,850,000 | 50% | " |
| 1/30 " " ..... | 3,500,000 | 60% | |
| 2/8 " " ..... | 5,310,000 | 90% | Occasional rods and diplococci |

* Injected same day with blood of anemic rat.

The hemoglobin drops more rapidly than the red cell count and returns more rapidly to normal.

### *2. Trypanosoma lewisi Infection Associated with Bartonella muris Anemia in Normal Albino Rats*

Mayer (7) noted the occasional appearance of *Bartonella muris* rods in the cells of mice and rats infected with trypanosomes.

In studies on *Trypanosoma lewisi* infection in adult albino rats we observed a moderate anemia during the first week of infection, with

the appearance in small numbers of rods and diplococci of the *Bartonella muris* bodies in the red cells. An effort was made to determine whether this anemia found in trypanosome infected rats is identical with the *Bartonella muris* anemia of splenectomized rats.

0.5 cc. of blood of an albino rat infected with *Trypanosoma lewisi* was injected into 3 week old rabbits. The trypanosomes died in the rabbit but a moderate anemia occurred within 10 days with the appearance of rods in the red cells. A small quantity of the blood of the anemic rabbit was injected into 30 gm. rats and an anemia developed in these rats with the appearance of the rods and cocci in the red cells. The virus of the anemia was thus transmitted through 30 gm. rats for several transfers. In this manner a strain of the virus of *Bartonella muris* anemia was isolated from adult nonsplenectomized albino rats infected with *Trypanosoma lewisi*.

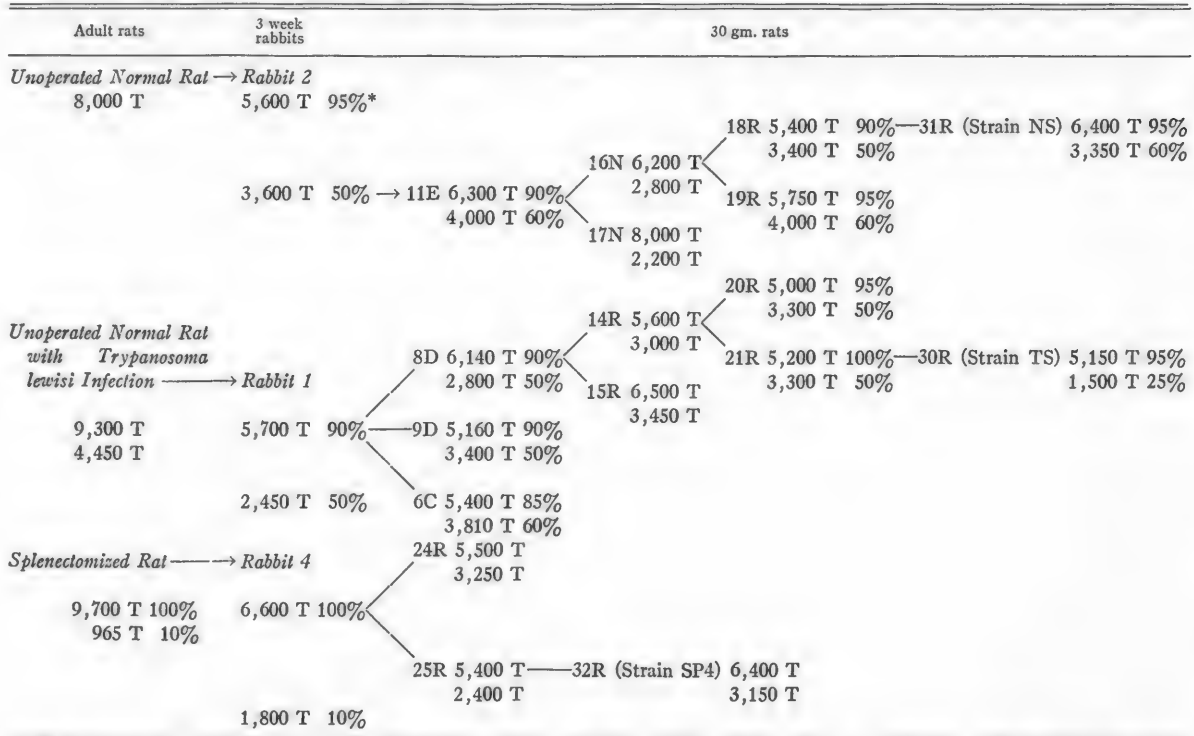
*Trypanosoma lewisi* infection in adult albino rats causes a profound disturbance in the function of the spleen and lowers the resistance of the rat to the virus of the *Bartonella muris* anemia. The deaths sometimes observed in young rats from *Trypanosoma lewisi* are due to the concomitant *Bartonella muris* anemia which in young rats is so often fatal. *Trypanosoma lewisi* infection causes a marked increase in the size of the spleen. The pulp cells become engorged with engulfed red blood cells and a marked disturbance in the function of these cellular elements is present. The effect is similar to that of splenectomy in the adult rat.

### *Latent Bartonella muris Anemia in the Adult Albino Rat*

It has been observed that the *Bartonella muris* anemia cannot be transmitted to adult animals with intact spleens by direct injection of blood from an infected animal. This infection can be transmitted, however, to normal young rats with intact spleens. This would indicate the probability of an infection early in life which results in a permanent immunity to the infection in the rat. This immunity is broken down with removal of the spleen and permits a second invasion with the virus. The rat often recovers, but repeated attacks may be produced either by the injection of whole blood from other anemic splenectomized rats or may occur spontaneously. It is possible however that the virus of anemia is present in the system from an infection in early life, and remains latent in the adult albino rat. An effort was

*The Isolation of Bartonella muris Virus from Trypanosome Infected Unoperated Adult Rats and from Normal Unoperated Adult Rats (Sample Protocols)*

The letter "T" is used in place of the last three zeros in the red cell count. The hemoglobin is expressed in percentages as calculated from Dare hemoglobinometer readings.




All the young rats showed *Bartonella muris* bodies in the red cells after injection with blood of the other animals.

* Only the red blood cell count before injection and lowest count after injection indicated.

made to determine whether or not a latent infection with the *Bartonella muris* virus is present in the normal adult unoperated rat.

Repeated examination of smears of the blood of normal rats failed to reveal the presence of the *Bartonella muris* bodies in the red cells. It was found that intravenous injection of 0.5 cc. of the blood of a normal adult rat into young rabbits produced anemia with the appearance of the *Bartonella muris* bodies in the red cells. The transfer of 0.5 cc. of the blood of anemic young rabbits intraperitoneally into young rats is followed by a severe anemia in these rats with the appearance of *Bartonella bodies* in the red cells. The anemia can be passed through successive transfers in young rats. The blood of a normal rabbit produces no effect when injected into young rats. The blood either of young or of mature normal rats produces no effect when injected into other young rats.

By the passage of whole blood of normal adult rats through young immature rabbits the virulence of the virus of the latent infection with the *Bartonella* anemia is sufficiently enhanced to produce the infectious anemia in young rats. It may be that the virus increases in quantity in the rabbit sufficiently to produce an anemia when the blood is subsequently injected into the rat.

### *The Effect of Splenectomy in Young Rats*

The adult normal rat is a carrier of the *Bartonella muris*. Further efforts were made to determine at what time in the life cycle of the rat it becomes a carrier of the virus of the *Bartonella muris* anemia.

Six 3 week old suckling rats were splenectomized. These young rats were placed in sterilized cages, completely isolated from the stock rats. None of these rats developed *Bartonella muris* anemia. It is evident then that during the first weeks of life the rat does not harbor the virus of *Bartonella muris* anemia, and therefore removal of its spleen is not followed by the infectious anemia. Since this period corresponds to the only period of life during which the *Bartonella muris* anemia may be transmitted in normal young rats by the injection of blood from an anemic splenectomized adult rat, it is not probable that the failure of splenectomized young rats to develop spontaneously the *Bartonella muris* anemia is due to a temporary protective immunity conferred from the mother. It would further suggest that the transmission of the virus from rat to rat is not necessarily through an insect host.

These experiments indicate that the *Bartonella muris* infection is latent in the adult normal rat. These rats are carriers of the virus and from their blood the virus can be isolated by passage through young rabbits. The invasion of the rat with this virus must occur after the suckling period, since suckling rats do not harbor the virus. The experiments further suggest a difference in the function of the spleen in young and adult rats.

## DISCUSSION

From the data presented it can be concluded that the adult rat is a carrier of the virus of *Bartonella muris* anemia. The failure to produce the picture of *Bartonella muris* anemia in other adult animals by removal of the spleen may be due to the fact that these animals are not carriers of the virus. The normal adult rat cannot be given the anemia by direct injection of blood from an anemic splenectomized rat, since the adult rat possesses a high degree of resistance, presumably from a transient infection in early life. During the suckling period the young rat does not harbor the virus of the anemia. Therefore removal of the spleen in these rats is not followed by *Bartonella muris* infection.

The spleen is physiologically and anatomically injured in many protozoan infections as in trypanosomiasis, leishmaniasis, spirochetosis and piroplasmic diseases and malaria. The spleen plays an important rôle in resistance of the organism to *Bartonella* infections. Severe injury to the spleen may bring about the same condition as splenectomy. In many mammals *Bartonella* and *Grahamella* infections accompany protozoan infections. It has been suggested by Bayon (8) that the *Grahamella* is capable of invading the organism as a result of injury to the spleen by a hematozoic infection. *Grahamella* infections are not observed in rodents under other conditions. From our work on *Bartonella muris* infection in rats, however, it is evident that the *Bartonella* anemia in the adult rat occurs only after an immunity to this infection acquired in early life is broken down by injury to or removal of the spleen. The spleen is the important factor in the maintenance of the resistance. No doubt *Grahamella* infections occur under similar circumstances.

*Bartonella bacilliformis* infection in man is a disease resembling the *Bartonella* and *Grahamella* infections of rodents. Oroya fever is a systemic infection charac-

terized by profound anemia and the occurrence of *Bartonella* bodies in the red cells. Verruga peruana is a skin infection without anemia and in this no *Bartonella* bodies occur in the red cells but they are found in the endothelial cells in the skin lesion. Noguchi (9) and Mayer and Kikuth (10) have demonstrated the common etiological factor in these two human diseases. It is of importance to determine what factors in the individual are responsible for the occurrence of one type or the other. From the observations in the literature in analogous infections and from our own work with *Bartonella muris*, an explanation presents itself. Verruga peruana may be a first infection in an individual with a normal spleen. Oroya fever may be a recurrence of an early infection or a flaring up of a latent infection in an individual with an injured spleen. Chronic malaria is a very common concomitant infection in individuals with Oroya fever. Such a chronic protozoan infection causes profound physiological injury to the spleen and may thus be responsible for the breaking down of the resistance of the individual to the *Bartonella* infection. Noguchi (11) made an effort to demonstrate the effect, if any, of malaria and of splenectomy on the course of infection with *Bartonella bacilliformis* in monkeys. From several observations he concluded that both malaria and *Bartonella bacilliformis* may coexist without unfavorable effect of one disease upon the course of the other, that splenectomy led to no appreciable aggravation of the *Bartonella* infection and that both malaria and splenectomy in the same animal had no appreciable effect on experimental verruga infection. The experiments, however, were not comprehensive enough to establish the relationship of chronic malarial infection to Oroya fever, nor do they disprove the conception that the spleen plays an important rôle in the latter disease.

Suggestive is the single observation made by Noguchi on a chimpanzee infected with *Bartonella bacilliformis*. A severe anemia developed in this animal only after an accidental infection with Rocky Mountain fever by the bite of the *Dermacenta andersonii* and disappeared only when the blood became negative on culture.

Kikuth (12) reports an increase in the number of "takes" of verruga peruana from 50 to 100 per cent following splenectomy in monkeys inoculated with material from human verruga papulae. In one instance typical Oroya fever with severe anemia developed in one of the monkeys that previously had been injected with verruga material but that had failed to develop lesions and subsequently was reinjected with such material.

These observations and our own in *Bartonella* infections in rats offer a possible explanation of the modus operandi of *Bartonella* infections in human beings. A first infection with *Bartonella bacilliformis* may result in verruga peruana or may have no clinical manifestations. Subsequent injury to the spleen by a chronic protozoan infection breaks down the protective mechanism of immunity to this organism with the occurrence of a systemic infection with the *Bartonella bacilliformis* in Oroya fever.

SUMMARY

1. The virus of *Bartonella muris* anemia of splenectomized rats may be transmitted to normal young unoperated rats and rabbits. This confirms the observations of Ford and Eliot.

2. *Trypanosoma lewisi* infections in normal adult rats are accompanied by an anemia most marked at the height of the infection and the appearance of *Bartonella muris* bodies in the red blood cells.

3. In young rats *Trypanosoma lewisi* may produce death from the severity of the anemia, complicating the disease. The anemic virus may be separated from the *Trypanosoma lewisi* infected blood by passage through young rabbits with subsequent maintenance of the strain in immature rats.

4. A strain of the virus of *Bartonella muris* anemia capable of producing an anemia in young rabbits and young rats for successive transfers has been isolated from the blood of normal adult unoperated rats by passage through young rabbits.

5. The adult normal rat is a carrier of the *Bartonella muris* virus.

6. Splenectomy in young suckling rats separated from the mother is not followed by a *Bartonella muris* anemia. The young suckling rat is not a carrier of the infection.

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# STUDIES ON BARTONELLA MURIS ANEMIA OF ALBINO RATS*

## III. The Protective Effect of Autoplastic Splenic Transplants on the Bartonella muris Anemia of Splenectomized Rats

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Plate 6

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Removal of the spleen in the adult albino rat is followed within 6 to 10 days by a severe progressive anemia which is fatal in 20 to 25 per cent of rats (1, 2, 3). In our laboratory approximately 100 per cent of splenectomized rats develop this anemia. Unsuccessful attempts have been made by investigators to protect rats from the anemia following splenectomy by feeding or injecting splenic tissue (Ford (4)) and by feeding large amounts of liver tissue (5). Vedder (5) attempted to transplant portions of spleen into the tunica vaginalis of the testes but was unsuccessful.

The first successful autotransplantation of splenic tissue in animals was made by Marine and Manley (6). They found that subcutaneous or intramuscular autoplastic spleen transplants in the rabbit are successful in almost 100 per cent of instances. Age is an important factor in the growth of the transplant. The younger the rabbit, the more rapid the growth of the transplant. Removal of the spleen is a powerful stimulus to the growth of the transplants. In mature rabbits the transplants took but usually did not grow and were often slowly resorbed. From their observations they concluded that in the rabbit the spleen is most important in early life. After sexual maturity it is either unimportant or its function may readily be assumed by other tissues (hematopoietic).

They have demonstrated that whereas anatomically the spleen is a highly complex structure, biologically, all the major elements of the spleen are simple as indicated by the uniform and marked regenerative capacity of their transplants.

The experiments reported in this communication were undertaken to determine: (a) Whether autoplastic transplants of splenic tissue

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in the adult rat regenerate, (b) what protective effect such autoplastic transplants of splenic tissue would have against the *Bartonella muris* anemia, and (c) the minimum amount of spleen left *in situ* that would protect the rats against this anemia.

The experiments were divided into three groups. In the first group autoplastic transplants of small pieces of splenic tissue were made in the abdominal wall of eight rats and 4 weeks later the spleen was removed. Three splenectomized rats in which no transplants had been made were used as controls.

### *Methods*

In this work mature albino rats about 3 months of age were used. These rats were raised in our laboratory from Wistar Institute stock. They were approximately uniform in weight. The methods for preparation of the animals for transplantation have been described in a previous communication (7).

The spleen was exposed through a small incision in the abdominal wall and one pole was gently pulled up into the opening. A piece was cut off for transplantation. Pieces of spleen about 6 mm. in diameter were immediately placed in small prepared pockets in the abdominal wall. Great care was exercised to avoid hemorrhage into these pockets and if hemorrhage occured, a new pocket was made. The sites of the transplants were indicated by the closing of the mouth of the pocket with a black silk suture. Four transplants were usually inserted into each animal. 4 weeks later the abdominal cavity was again opened, the mesentery of the spleen ligated and the spleen removed. The peritoneum and skin were closed. Red blood cell counts and hemoglobin determinations were made at frequent intervals.

The results of this experiment (tabulated in Table I) indicate that transplants made 4 weeks before splenectomy afford no protection against *Bartonella muris* anemia. Histological examination of the transplants of Rat 1082 which died at the height of the anemia revealed replacement of the plant by reticular cells that formed large sinusoid spaces filled with red cells. Degenerative changes of the reticular elements and areas of necrosis were present. The splenic tissue, both pulp and follicles, had not regenerated at this time. The reticulum present, however, showed those same changes of degeneration and focal necrosis found in the spleen of young rats with *Bartonella muris* anemia. It is of interest that the transplant though not regenerated, reacted to the infection in the same way as the intact spleen. Marine and Manley (7) observed that in rabbits dying of pneumonia the

spleen transplants on section were soft, engorged with blood and microscopically showed an increase in pulp cells. These changes are the same as those found in the intact spleen in the presence of infection. Five rats were not killed until 3 months after the transplants had been made. Upon examination the transplants were very large in size. It is evident that complete regeneration of both the pulp cells and Malpighian corpuscles of the transplants had occurred within 3 months. In the absence of the spleen there was a marked hypertrophy of the regenerated transplants. These transplants were really small spleens. They were not regenerated, however, at the time the spleen was removed and did not prevent the development of *Bartonella muris* anemia (Table I).

In the second group of experiments nine adult albino rats were used. In seven of these, autoplastic splenic transplants were made 7 weeks prior to splenectomy. In three rats the spleen alone was removed.

The method of transplantation has been described above. In these rats two transplants were inserted on either side of the midline in the sheath of the rectus muscle. Seven weeks later the spleen was removed.

In this group four of the seven rats with transplants did not develop an anemia and failed to show *Bartonella muris* bodies in the red cells. More than half the rats were protected by autoplastic spleen transplants. Three weeks after the splenectomy or 10 weeks after the transplantation the rats were killed with ether and the transplants carefully removed and sectioned (see Table II).

Comparative histological studies of the transplants in the protected and unprotected rats reveal a striking contrast. In those rats that were unprotected the Malpighian corpuscles of the transplants are completely regenerated, but the entire pulp is filled with red blood cells. The corpuscles stand out strikingly against the surrounding intense congestion (Fig. 2). Large sinusoids are present but are distended with blood and the endothelium flattened. In those rats that were protected the transplants are smaller, but both the pulp cells and the Malpighian corpuscles are completely regenerated and have the appearance of adult splenic tissue (Fig. 3). In the transplants that protected, large amounts of hemosiderin pigment were present in the

*Effect of Spleen Autotransplants Made 4 W*

| No. and sex | Weight | Date of transplant | Interval between transplant and splenectomy | Blood count before splenectomy | Counts after splena | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 1 | 3 | 5 | 6 | 7 | 9 | |
| | *gm.* | | | | | | | | | | |
| 1078 M | 215 | 10/6 | 4 weeks | 90% 9,600 T | 80% 8,900 T | 81% 8,350 T | 45% 3,550 T | 44% 4,150 T | 45% 4,100 T | 60% 5,050 T | 7 7,7 |
| 1079 M | 203 | " | " | 95% 9,600 T | 85% 8,550 T | 78% 9,800 T | Died | | | | |
| 1080 M | 230 | " | " | 90% 8,500 T | 95% 9,500 T | 90% 9,450 T | 28% 1,450 T | 34% 3,900 T | 25% 4,100 T | 42% 3,550 T | 6 4,8 |
| 1081 M | 203 | " | " | 90% 9,300 T | 81% 7,500 T | 85% 8,250 T | 32% 4,100 T | 26% 2,400 T | 35% 3,700 T | 50% 4,400 T | 7 7,6 |
| 1082 M | 203 | " | " | 100% 11,200 T | 75% 9,000 T | 75% 7,950 T | 40% 4,900 T | Died | | | |

The letter "T" is used in place of the last three zeros in the red cell count. The hemog

*to Splenectomy on Bartonella muris Anemia*

| ber of days | | | | | | | | 1/23/30 |
|---|---|---|---|---|---|---|---|---|
| 13 | 16 | 20 | 24 | 27 | 34 | 37 | 45 | 55 | Histology of transplants |
| %<br>00 T | 80%<br>7,500 T | 90%<br>8,900 T | 90%<br>8,650 T | | | | | | Fully regenerated splenic tissue with prominent follicles and normal pulp tissue. Pigment in endothelial cells |
| %<br>50 T | 75%<br>4,800 T | 70%<br>4,600 T | 8,150 T | 80%<br>7,150 T | 6,800 T | 4,000 T | 4,200 T | 4,800 T | Small spleen with prominent Malpighian corpuscles and congested pulp. Erythrophagocytosis pulp cells present. Sinuses seem to be present and filled with red blood cells |
| | | | | | | | | | 11/9/29<br>Malpighian corpuscles are not present. Pigment in cells of capsule. Transplant replaced by reticulum showing marked swelling and necrosis of cells with occasional cell containing pigment. Small foci of necrosis are present as are seen in spleen of young anemia rats |

pressed in percentages as calculated by the Dare hemoglobinometer.

TABLE

| No. and sex | Weight | Date of transplant | Interval between transplant and splenectomy | Blood count before splenectomy | Counts after splene | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 1 | 3 | 5 | 6 | 7 | 9 | |
| | *gm.* | | | | | | | | | | |
| 1083 F | 189 | 10/6 | 4 weeks | 98% 9,800 T | 72% 7,500 T | 70% 6,150 T | 15% 3,300 T | 12% 1,900 T | 10% 2,100 T | Died | |
| 1084 F | 179 | " | " | 98% 9,750 T | 82% 8,750 T | 78% 9,750 T | 20% 2,370 T | 22% 2,150 T | 10% 2,050 T | 18% 2,150 T | 3 2,0 |
| 1085 F | 170 | " | " | 93% 10,010 T | 85% 9,200 T | 87% 7,200 T | 39% 2,800 T | 30% 2,800 T | 38% 2,500 T | 42% 4,500 T | 6 5,9 |
| 1086 M | 205 | None | Control splenec. | 96% 10,000 T | 89% 7,300 T | 85% 7,225 T | 48% 3,600 T | 26% 1,700 T | 15% 2,300 T | Died | |
| 1087 M | 217 | " | " | 94% 9,800 T | 67% 9,650 T | 69% 7,000 T | 78% 5,850 T | 88% 6,950 T | 45% 6,100 T | Died | |
| 1088 F | 180 | " | " | 90% 10,030 T | 87% 5,800 T | 80% 6,500 T | 35% 5,000 T | 23% 2,700 T | 18% 1,700 T | 22% 2,650 T | 4 2,5 |
| 1089 F | 162 | Normal control | | 90% 8,000 T | 95% 8,550 T | 100% 8,450 T | 95% 9,600 T | 100% 9,550 T | 85% 7,000 T | 85% 8,150 T | 10 8,5 |

reticular cells of the pulp. In those that did not protect, though there was so striking a quantity of blood present and though the rats had had severe anemia, no hemosiderin pigment was deposited in the few cells of the pulp. The reticular cells of the splenic pulp had undergone an exhaustion destruction that exceeded the rate of regeneration.

*ded*

| er of days | | | | | | | | 1/23/30 |
|---|---|---|---|---|---|---|---|---|
| 16 | 20 | 24 | 27 | 34 | 37 | 45 | 55 | Histology of transplants |
| 75%<br>3,900 T | 70%<br>4,100 T | 4,800 T | | 80%<br>7,150 T | 6,000 T | 80%<br>6,100 T | 75%<br>6,600 T | Malpighian corpuscles prominent, stand out against intense congestion about them in pulp. Endothelial and reticular elements as found in normal spleen are seen |
| 80%<br>6,300 T | 85%<br>6,500 T | 6,600 T | | 6,700 T | 6,250 T | 6,150 T | 6,200 T | Malpighian corpuscles prominent. Pulp intensely congested. Erythrophag prominent. Pigment present |
| 70%<br>4,550 T | 5,100 T | 8,600 T | 75%<br>6,450 T | 5,500 T | 5,650 T | 5,300 T | 5,900 T | |
| 85%<br>8,600 T | 85%<br>8,750 T | 7,200 T | 8,200 T | 7,500 T | 7,800 T | 7,500 T | 8,600 T | |

In the third group, an effort was made to determine the minimum amount of spleen left *in situ*, that would prevent the development of the anemia. Eight adult rats were used. In two rats one-quarter of the spleen was removed, in two one-half of the spleen was removed, in two three-quarters of the spleen was removed and in two the entire spleen was removed.

| No. and sex | Weight | Date of transplant | Interval between transplant and splenectomy | Count before splenectomy | Counts after splenectomy—days | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 1 | 3 | 4 | 5 | 6 | 8 |
| | gm. | | | | | | | | | |
| 1204 F | 110 | 11/23/29 | 7 weeks | 108% 8,500 T | 105% 8,500 T | 95% 8,000 T | 108% 7,300 T | 110% 8,000 T | 100% 9,100 T | 100% 8,310 T |
| 1205 F | 133 | " | " | 110% 8,700 T | 110% 8,500 T | 95% 8,200 T | 103% 8,160 T | 98% 7,500 T | 90% 7,050 T | 85% 7,210 T |
| 1206 F | 135 | " | " | 110% 8,150 T | 110% 8,150 T | 100% 8,500 T | 110% 7,680 T | 110% 8,000 T | 108% 8,050 T | 110% 8,000 T |
| 1207 F | 131 | " | " | 108% 8,800 T | 108% 8,600 T | 100% 8,000 T | 110% 7,230 T | 100% 7,500 T | 108% 8,750 T | 110% 8,950 T |
| 1208 F | 108 | " | " | 108% 8,400 T | 108% 8,150 T | 80% 5,400 T | 40% 1,830 T | 18% 1,500 T | 25% 1,200 T | 45% 2,350 T |
| 1209 M | 115 | " | " | 110% 7,500 T | 110% 7,500 T | 80% 7,000 T | 100% 7,230 T | 20% 2,500 T | 20% 1,750 T | 35% 2,100 T |
| 1210 M | 152 | " | " | 106% 7,800 T | 106% 7,700 T | 60% 4,000 T | 33% 1,300 T | 25% 1,500 T | 20% 1,150 T | 20% 1,000 T |
| 1211 M | 136 | None | Control splenec. | 109% 9,000 T | 109% 9,700 T | 80% 4,500 T | 40% 2,060 T | 22% 1,200 T | Dead | |
| 1212 M | 152 | " | " | 105% 9,800 T | 110% 9,650 T | 90% 7,000 T | 40% 1,850 T | 35% 1,500 T | Dead | |
| 1213 M | 141 | " | " | 105% 9,800 T | 100% 9,700 T | 70% 7,500 T | 20% 1,050 T | 20% 1,100 T | 10% 960 T | Dead |
| 1214 M | 136 | Control normal | | 110% 8,000 T | 110% 7,600 T | 90% 7,500 T | 108% 7,550 T | 115% 8,000 T | 105% 8,600 T | 115% 9,850 T |

The letter "T" is used in place of the last three zeros in the red cell count. The hemo

* In all the transplants a connective tissue capsule separates the transplant from the sur

| 17 | 20 | 25 | Autopsy findings† | Histology of the transplants |
|---|---|---|---|---|
| 100% 8,200 T | 110% 8,600 T | 95% 8,600 T | No evidence of anemia<br>Small transplants | * Splenic elements are all present. Follicles are distinct. Pulp cells are regenerated and have large amounts of hemosiderin pigment in their protoplasm. Sinuses contain blood elements. The usual giant cells are present |
| 92% 8,900 T | 95% 8,800 T | 110% 8,400 T | Two transplants, very large, two resorbed | All elements regenerated and hypertrophied. Follicles are prominent. Pulp cells show hemosiderin pigment. Both endothelial and reticular elements are prominent |
| 100% 9,100 T | 90% 8,500 T | 110% 8,200 T | Two transplants found, 3 to 4 mm. each | Small transplants. Connecting tissue septum filled with hemosiderin pigment. Follicles small. Pulp cells regenerated and filled with pigment. Central arteries are small |
| 90% 9,350 T | 110% 9,100 T | 90% 9,200 T | Two transplants, 3 to 4 mm.<br>Two large transplants, 1 cm. in diameter | Small transplants but all elements regenerated. Hemosiderin pigment in the pulp cells. Giant cell occasionally seen as in adult rat spleen. Arterioles small. Reticular and endothelial cells large and prominent |
| 80% 5,200 T | 85% 5,250 T | 80% 6,200 T | Two transplants, large, 1 cm. and 1.5 cm.<br>Two absorbed | Malpighian corpuscles stand out prominently. Vessels small. Pulp consists of large masses of blood, filling irregular sinuses lined with flat endothelium. There are only scattered reticular and endothelial elements resembling normal spleen. The pulp cells are strikingly absent |
| 80% 5,850 T | 85% 6,300 T | 100% 7,500 T | Three large transplants, 1.5 cm. in diameter | Transplant similar to 1208. Malpighian corpuscles are prominent. Pulp tissue replaced by red blood cells. Endothelial elements are few and show no pigment |
| 90% 4,200 T | 100% 3,750 T | 80% 5,100 T | Four large transplants, 1.5, 1.4, 0.8 and 0.8 cm. in diameter | Large transplants. Striking contrast between regenerated follicles and the blood pulp tissue. The sinuses are distended with blood. Endothelium is thin. Reticular cells very sparse |
| 98% 9,400 T | 100% 8,600 T | 95% 9,100 T | | Control normal |

pressed in percentages as calculated by the Dare hemoglobinometer.
uscle. † All rats killed 74 days after transplantation.

TABLE III

*The Effects of Removal of Varying Quantities of Splenic Tissue*

| No. and sex | Weight | Amount of spleen removed | Blood count before operation | Counts after operation—number of days | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 3 | 4 | 7 | 8 | 11 | 13 | 16 | 21 | 27 | 42 |
| | *gm.* | | | | | | | | | | | | |
| 1376 | 225 | ½ | 100% | 105% | 95% | 98% | 100% | 110% | | | 90% | | |
| M | | | 9,000 T | 8,500 T | 8,350 T | 8,100 T | 8,400 T | 8,200 T | 8,500 T | 8,600 T | 8,050 T | | |
| 1377 | 210 | " | 88% | 95% | 100% | 110% | 110% | 115% | | | 100% | | |
| M | | | 7,700 T | 7,000 T | 7,500 T | 7,550 T | 7,600 T | 8,400 T | 7,700 T | 8,100 T | 8,000 T | | |
| 1378 | 250 | " | 90% | 95% | 100% | 100% | 115% | 112% | | | 110% | | |
| M | | | 8,500 T | 8,750 T | 9,500 T | 7,200 T | 7,200 T | 8,500 T | 8,700 T | 8,500 T | 9,200 T | | |
| 1380 | 230 | ¾ | 105% | 100% | 110% | 95% | 100% | 115% | | | 105% | | |
| M | | | 9,200 T | 9,000 T | 8,950 T | 8,500 T | 8,700 T | 9,400 T | 9,000 T | 9,400 T | 9,400 T | | |
| 1381 | 252 | " | 110% | 100% | 105% | 110% | 100% | 115% | | | | | |
| M | | | 9,250 T | 8,900 T | 8,900 T | 8,600 T | 8,800 T | 8,500 T | 8,900 T | 8,500 T | 9,000 T | | |
| 1382 | 210 | " | 103% | 103% | 100% | 110% | 100% | 115% | | | | | |
| M | | | 9,700 T | 9,200 T | 8,750 T | 8,400 T | 8,700 T | 9,200 T | 9,000 T | 8,700 T | 9,400 T | | |
| 1072 | 243 | Entire | 100% | | | | | | | | | | |
| M | | | 8,200 T | 5,200 T | 890 T | Died | | | | | | | |
| 1073 | 267 | " | 105% | | | | | | | | | 80% | 78% |
| M | | | 7,500 T | 5,490 T | 2,030 T | 1,460 T | | 1,960 T | 2,000 T | 3,820 T | 4,500 T | 7,300 T | 8,450 T |
| 1074 | 252 | " | 100% | | | | | | | | | 60% | 70% |
| M | | | 7,900 T | 5,400 T | 3,230 T | 1,730 T | | 2,080 T | 2,900 T | 2,790 T | 4,200 T | 5,250 T | 7,000 T |

The letter "T" is used in place of the last three zeros in the red cell count. The hemoglobin is expressed in percentages as calculated from readings with the Dare hemoglobinometer.

The peritoneal cavity was exposed by an incision in the upper left quadrant of the abdominal wall, the spleen was gently drawn out of the cavity, a ligature inserted through the mesentery and tied about that portion of the mesentery corresponding to the part of the spleen that was removed. Where the entire spleen was removed a single ligature was tied close to the hilum about the mesentery. The bleeding was slight. The splenic stump was gently replaced in the cavity, care being taken not to tug on the mesentery, the peritoneum and the abdominal wall sutured by continuous sutures. Red blood cell counts before the operation and at frequent intervals after the operation were made.

From Table III it is evident that a small amount of splenic tissue left *in situ* is sufficient to protect the rat against the *Bartonella muris* anemia. This finding is in agreement with Vedder who observed that less than half the spleen will protect against the anemia.

## DISCUSSION

A comparative study of the splenic transplants in rats and rabbits indicates a striking difference in the regenerative capacity of the transplants. In the rabbit, Marine and Manley (6) showed that though splenic autotransplants will grow in the adult rabbit in the absence of the spleen in many instances, these transplants are often resorbed or remain small and do not undergo hypertrophy. In the rat, however, we found that splenic autotransplants will grow even in the presence of the spleen and in its absence will undergo marked hypertrophy, growing to the size of accessory spleens or larger. This indicates a difference in the function of the adult spleen of the rat and rabbit.

The observations reported in this paper tend to indicate that the pulp cells are the responsible factor for the specific protective mechanism of the spleen in the rat against the *Bartonella muris* anemia. We have an accurate gauge of physiological activity of the pulp cells of the spleen.

It has been shown in a previous communication (7) that *Trypanosoma lewisi* infection in the adult normal albino rat is accompanied at the height of the infection by a moderate anemia and the appearance in the red blood cells of *Bartonella muris* bodies. Studies on the pathology of this infection indicate that the most significant changes occur in the spleen. At the height of the infection the spleen may enlarge six to ten times. The follicles increase in size and the pulp

cells become engorged with engulfed red blood corpuscles. It is probable that this process of phagocytosis impairs or destroys the normal physiological activity of this group of cells and produces the same effect on the resistance of the organism to the *Bartonella muris* anemia as splenectomy. These observations indicate a specific function of the reticular cells of the spleen.

SUMMARY

Autoplastic splenic transplants were made in adult albino rats 4 weeks and 7 weeks prior to splenectomy and the protective effects against infection with the *Bartonella muris* anemia observed.

1. One-fourth of the spleen left *in situ* will protect adult albino rats against the *Bartonella muris* anemia.

2. Autotransplantation of splenic tissue in adult rats is successful in over 90 per cent of instances.

3. Autoplastic splenic transplants performed 7 weeks prior to splenectomy afford protection against *Bartonella muris* anemia in more than 50 per cent of instances, whereas 4 week old transplants do not protect.

4. A comparative histological study of the transplants of protected and unprotected rats reveals a regeneration of the pulp cells in the protected rats and an exhaustion destruction of the pulp in the unprotected rats.

5. The reticular cells play a specific rôle in protecting the adult albino rat against *Bartonella muris* anemia.

We wish to thank Dr. David Marine for his helpful advice throughout the course of this work.

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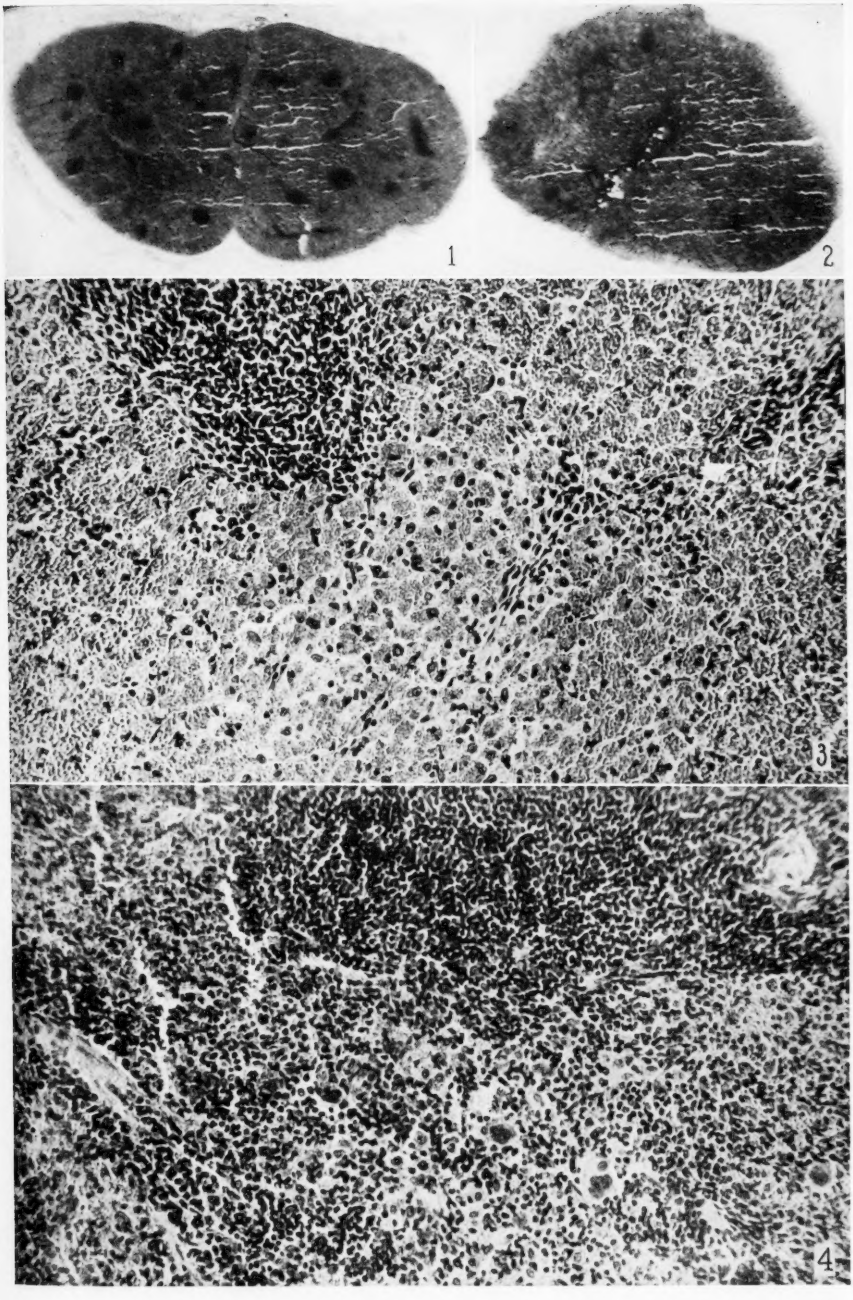
## EXPLANATION OF PLATE 6

FIG. 1. Rat 1208. Autoplastic splenic transplant performed 7 weeks prior to splenectomy. M × 30. This transplant did not afford protection against *Bartonella* anemia following removal of the spleen. The follicles stand out prominently against the blood filled pulp tissue.

FIG. 2. Rat 1205. Autoplastic splenic transplant performed 7 weeks prior to splenectomy. M × 30. This transplant protected the rat against the *Bartonella muris* anemia following the removal of the spleen. Note the cellular appearance of the pulp.

FIG. 3. Rat 1208. Autoplastic splenic transplant. M × 240 (higher magnification of Fig. 1). Shows exhaustion degeneration of the pulp cells and complete regeneration of follicle.

FIG. 4. Rat 1205. Autoplastic splenic transplant. M × 240 (higher magnification of Fig. 2). Complete regeneration of pulp tissue.




(Perla and Marmorston-Gottesman: *Bartonella muris* anemia, III)

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